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COMPARISON OF SOME PHYSICOCHEMICAL PROPERTIES OF GLUTEN FROM HARD, SOFT AND DURUM WHEATS

by

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ABSTRACT

Physicochemical methods were used in an attempt to differentiate glutens from hard, soft and durum wheats. Intrinsic viscosity determinations of samples prepared under different conditions, sulfhydryl group analysis and sedimentation velocity analysis of gluten treated with performic acid indicated the involvement of the disulfide group in determining the physical properties of glutens from the three classes of wheat.

Diffusion studies, molecular weight determinations and distribution of sedimentation coefficient studies indicate very small differences in the mean particle size.

By far the greatest difference in the behavior of the glutens from the three wheats was shown after performic acid oxidation. Gluten from durum showed a large decrease in the sedimentation coefficient, while the gluten from hard wheat showed the smallest decrease. The experimental data do not permit definite conclusions but it is suggested that one of the factors contributing to this difference may be the number of intermolecular disulfide bonds in gluten.

Introduction

It is well known that flour from hard wheat is used primarily for bread making, soft wheat flour for cakes and pastries, and durum wheat flour for alimentary pastes. Each flour is suited for a specific purpose because of the differences in the physical properties of dough made from each flour. One of the factors which contributes to these differences is believed to be the gluten, in which form the bulk of the wheat protein occurs.

Little is known of the essential differences among these flours from the three classes of wheat. Insufficient work has been done to determine whether the physical properties of dough and gluten can be accounted for in terms of the chemical groupings of the molecules or of the spatial arrangements of the constituents. Most physicochemical studies on wheat proteins have been done on proteins from hard wheat. Very few reports have been published on comparative studies of glutens from hard, soft and durum wheats.

The present studies, using physicochemical methods, were undertaken in an attempt to find differences in chemical and/or physical properties of the three glutens.

LITERATURE REVIEW

Gluten solubility and heterogeneity

Fundamental studies on the constitution of wheat proteins were initiated by Osborne (77). Wheat gluten was concluded to be essentially an intimate mixture of two separate and distinct individual proteins, gliadin and glutenin. Gliadin was separated by its solubility in 70% alcohol, while glutenin was soluble only in dilute alkali or acid. Because gluten dispersed in dilute alkali more readily, most workers used this solvent. However, the apparent deleterious effect of alkali caused investigators to search for a better solvent.

Since Osborne's time, cereal chemists have devoted considerable effort in an attempt to find a suitable solvent for studying gluten which, unfortunately, is insoluble in the common buffer systems used for many other proteins. The question of denaturation is inevitably raised upon solubilization in a specific solvent. Denaturation has been defined as "a process in which the spatial arrangement of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement" (53). If this definition is accepted, then denaturation is unavoidable in gluten solubilization, and therefore, this aspect will not be considered in this survey.

Blish and Sandstedt (8) reported that a glutenin fraction, obtained by using dilute acetic acid, was different in physical properties and chemical composition from that prepared using alkali. Later, the same workers(89) fractionated gluten from dilute acetic acid dispersion into three fractions which they designated as glutenin,

gliadin and mesonin, representing 20 - 25%, 45 - 55% and about 25% respectively of the gluten.

Cook and Alsberg (21) reported a new method of preparing glutenin by first dispersing gluten in 30% urea, then precipitating the glutenin by adding salt or by dilution. Concentrated urea, besides dispersing gluten completely, was believed to avoid the alterations caused by prolonged contact with alcohol and alkali. Cook (20) by fractional precipitation of gluten from 30% urea concluded on the basis of solubility that there was no clear-cut difference between gliadin and glutenin. This was one of many observations which contradicted Osborne's original classification of wheat proteins.

Complete dispersion of gluten was achieved by Cook and Rose (22) in sodium salicylate solutions. It was found that the minimum concentration of salicylate necessary for total dispersion was 8%. McCalla and Rose (62) using this dispersing medium, fractionally precipitated gluten by adding magnesium sulfate. When the amount of gluten precipitated was plotted against the concentration of magnesium sulfate, a smooth curve was obtained indicating that gluten is not composed of distinct fractions, glutenin, gliadin and mesonin. It was suggested that gluten, except the most soluble 10 - 15% not precipitated by half-saturated magnesium sulfate, is a single protein complex which can be divided into many fractions differing systematically in both physical and chemical properties. This view was further substantiated by the study of fractional solubility of gluten in sodium salicylate reported by Spencer and McCalla (97).

Harris (38,39) dispersed gluten from hard and durum wheats in 10% sodium salicylate and fractionated according to the method of McCalla and Rose (62). A correlation between certain fractions and loaf volume was reported but nothing fundamental in characterizing these fractions was offered.

The results of a detailed study of gluten and its fractions in sodium salicylate by diffusion and sedimentation were reported by McCalla and Gralén (63). It was shown that gluten in salicylate solution existed in two states, molecularly dispersed and in aggregates. The fact that progressive variation in physical properties was observed in both states supported strongly the contention that gluten is a reversible protein complex as suggested earlier (62).

The work of Cunningham et al. (24) further gave evidence that gluten is not a mixture of a few distinct proteins. Formic acid extracts of glutens from wheat, oat, barley and rye flours were fractionally precipitated by the addition of a variety of salts as well as by changes in pH. A curve, similar to that of McCalla and Rose (62) was obtained by plotting the amount of precipitate against the salt concentration or pH.

Vercouteren and Lontie (110) reported that 2,4-dimethylformide dissolves 92% of the nitrogenous constituents of gluten at 20°C and 98% at 60°C. However, no report has been published using this solvent for basic studies of gluten. DeDeken and Mortier (27) studied the solubility of gluten in oxidising and reducing solutions made alkaline with sodium hydroxide. Reducing agents, thioglycolic acid, sodium sulfide, cysteine and thiophenol dispersed

about 90% of the gluten protein, while oxidising agents such as bromate, periodate and persulfate dispersed only 11 to 45%. On this basis, it was suggested that the sulfhydryl-disulfide system plays an important role in the basic structure of gluten and therefore, in solubility.

Electrophoretic studies of gliadin by Mills (69) indicated that gliadin was heterogeneous and composed of at least four components. A more detailed study by electrophoresis and fractionation of gluten was carried out by Jones et al. (50). Of the various buffer systems tried, aluminum lactate was considered most suitable since symmetrical peaks on ascending and descending limbs of the electrophoresis cell were obtained. It was reported that wheat gluten was composed of at least four major and one minor components. Partial separation of the electrophoretic components was achieved by precipitation of gluten from acid solution by increasing pH or ionic strength. A chromatographic procedure using carboxymethylcellulose columns was applied by Woychik et al. (116) to isolate purified gluten components in "preparative" amounts. On the basis of their electrophoretic behavior it was suggested that the five components separable on the column represent individual proteins.

The same procedure, i.e., separation on a carboxymethylcellulose column was used by Simmonds and Winzor (93). Seven components were separated. One of the major peaks, when rechromatographed, showed only one symmetrical peak but it was pointed out that this component may not be homogeneous when subjected to other methods of fractionation.

Fractionation of gluten by partial solubility in methanol-chloroform mixture and in dilute formic acid was reported by Meredith et al. (68). The five fractions thus obtained were subjected to various physicochemical analyses. Differences in fractions were found by viscosity measurements, amino acid analysis and by electrophoresis. As was expected on the basis of other fractionation studies, none of the fractions was electrophoretically homogeneous.

Starch gel electrophoresis was applied to wheat gluten by Woychik et al. (114). Aluminum lactate containing 3 M urea was used to separate nine components. Gliadin was found to contain eight components and glutenin was not separable into fractions. Glutenin remained almost completely at the origin in the starch gel, and after elution, was found to be electrophoretically homogeneous.

Viscosity

Cereal chemists have always been interested in the possibility of evaluating quality of flours by physical or chemical tests. Among the numerous tests devised, measurement of viscosity on flour-water suspensions was one of the earliest applied. Gortner and Doherty (35), on the basis of viscosity measurements, suggested that the basic difference in the glutens from strong and weak flours may be due to the difference in the size of the gluten particles and that "at least a part of the particles comprising the weak gluten may lie nearer the boundary between the colloidal and crystalloidal states of matter than is the case with the stronger glutens".

Various other investigators attempted to interpret viscosity measurements in terms of flour quality, among them, Rich (85) who by statistical analysis found a relationship between viscosity, total protein content, ash content and baking strength. A very large number of flour samples from 1930 and 1931 crops in western Canada was tested. A torsional type viscometer was used to measure the viscosity of flour-water suspensions. Only qualitative results were presented and interpretation of the data in terms of fundamental structure was not attempted.

Cook (20) determined the change in viscosity of gluten dispersed in 30% urea upon heat treatment. The various fractions, obtained by additions of magnesium sulfate, were studied and it was concluded that the glutenin fraction was first affected when subjected to elevated temperatures: next the gliadin fraction of low solubility and finally under severe conditions, all of the glia-

din was affected.

Rose and Cook (86) studied the viscosity of gluten dispersed in alkali, acid and neutral solvents. It was found that the viscosity of gluten dispersed in urea and in sodium salicylate was much more concentration-dependent than those dispersed in sodium hydroxide or acetic acid. When gluten was precipitated from each of the four solvents, there was an indication that the properties of gluten were affected more by alkali and acid than by the neutral solvents. There was a significant partial correlation between the quality of gluten and viscosities determined in urea and in sodium salicylate. The same workers (23) studied the effect of heat treatment on the viscosity of gluten in the same solvents. For dispersions in sodium hydroxide and in acetic acid the viscosity decreased gradually with temperature, whereas in urea and in salicylate a pronounced change was observed above 70°C. Interpretation of the data in terms of basic protein structure was not attempted.

Harris and coworkers (40-43) published a number of papers in an attempt to characterize glutens from different varieties and types of wheat using viscosity measurements. Viscosities of gluten dispersed in 10% salicylate were measured with an Ostwald viscometer. Gluten from hard red spring wheat was found to have the highest viscosity of the three types hard, soft and durum. Differences in viscosities were interpreted to be differences in the size of the protein molecules, as suggested earlier by Gortner and Doherty (35). A factor, which was called the specific volume of the gluten, was calculated using the equation

$$\frac{\eta}{\eta_o} = \frac{1 + 0.5\varphi}{(1 - \varphi)^4}$$

where η/η_o is the relative viscosity, and φ is the percentage of the system occupied by the volume of the disperse phase. φ/c is defined as the specific volume, where C is the concentration. The specific volumes were calculated to be 7.9 - 7.1 for hard wheats, 6.9 for soft and 5.8 for durum. There is obviously an error in their calculation. If the specific volume is defined, as they have done, as the volume occupied by one gram of protein, then, from these figures, the density of the protein can be calculated. However, calculations of density give values which are smaller by a factor of ten, e.g., the density for gluten from hard wheat is calculated to be 0.12. Colvin and McCalla (17) determined the density of gluten to be 1.310 g/cc. Thus, the significance of the values for specific volumes is questionable.

Later Geerdes and Harris (33) attempted to characterize gluten from hard red spring wheat and durum wheats, again by viscometry. The intrinsic viscosities were calculated to be 25.8 cc/g for hard wheat and 22.8 cc/g for durum wheat. Hydration factors of 10.3 for hard wheat, 9.0 for durum and axial ratios of 15.4 for hard and 14.0 for durum were calculated. Since these calculations for hydration and axial ratios are based on equations derived for particles of specific shapes, and since the molecular characteristics of gluten particles are not well defined, the figures presented can be taken only as an indication, and differences between the two wheats are probably not significant.

Barmore (6) fractionated gluten into components differing progressively in viscosity and solubility. Differences in viscosity were interpreted to indicate differences in axial ratios.

Approximate calculations of the axial ratios for the various fractions indicated values of 15 for the most soluble gliadin, 26 for the gliadin of low solubility, 41 for gluten and 47 for glutenin. This indicated a relationship between symmetry and solubility. These results were interpreted to add further evidence that there is no clear distinction between gliadin and glutenin.

Viscosities of wheat gluten in dilute acetic acid and in sodium salicylate were measured by Udy (109). Dispersions in dilute acetic acid exhibited a different concentration dependence than those in sodium salicylate, as found earlier by Rose and Cook (86). For dilute protein concentration in dilute acetic acid, the electrostatic effect was quite pronounced, as indicated by a rise in the reduced viscosity. Intrinsic viscosities calculated for both hard and soft wheats were the same, .309 dl/g in sodium salicylate. When reduced by the addition of bisulfite or thioglycolic acid, the intrinsic viscosity fell to .288 dl/g. On the other hand, for dispersions in dilute acetic acid, the intrinsic viscosity cannot be calculated unless the electrostatic effect is repressed, but upon addition of a reducing agent an intrinsic viscosity of .188 dl/g was obtained. It was suggested, on the basis of intrinsic viscosity data, for all wheat gluten, that the constituents of the complex which contributes to the viscous behavior of gluten dispersions are very nearly the same size.

Cluskey et al. (19) fractionated gluten from different varieties of wheat and determined the intrinsic viscosities. Small but statistically significant differences were found between the electro-

phoretically separated fractions obtained from hard and soft wheats. The mean intrinsic viscosity of hard wheat gluten was 13% higher than that of soft wheat, 0.35 dl/g to 0.31 dl/g. This difference was statistically significant and contrary to the results of Udy (109) who found no difference between the intrinsic viscosities of hard and soft wheat glutens.

Recently, Taylor and Cluskey (106) determined the intrinsic viscosities of gluten, glutenin and gliadin in sodium lactate of two ionic strengths. In 0.03 ionic strength solution the intrinsic viscosity of gluten was 0.26 dl/g, of gliadin 0.16, of glutenin purified four times 0.62 from Ponca wheat and 0.70 from Wichita wheat. When the ionic strength was decreased to 0.003, intrinsic viscosities increased to 0.57 for gluten, 1.14 for glutenin, 0.21 for gliadin and 2.23 for 4X glutenin from Wichita wheat. This increase in intrinsic viscosity can very well be due to the electrostatic effect because of low salt concentration or to the greater probability of aggregation.

Molecular weight determinations

One of the earliest attempts to calculate the molecular weight of gliadin was made by Ashmarin (3). It was assumed that each protein molecule contains one or more integral moles of the amino acids found in them. Taking the amino acid composition reported by Osborne (77), a molecular weight of 12,460 was obtained for gliadin.

Cohn et al. (16) calculated the minimal molecular weights of certain proteins on the basis of individual amino acid or sulfur content. A molecular weight of 20,700 was calculated for gliadin. On the basis of tryptophane and tyrosine content, a minimal molecular weight of 36,300 was derived for glutenin.

An ultracentrifugal study of wheat gliadin was made by Krejci and Svedberg (56). The protein was found to be heterogeneous with respect to molecular weight. By sedimentation equilibrium the molecular weight of the principal component in a certain pH and temperature range was calculated to be a mixture of whole and half molecules of 34,500 and 17,250. At higher temperatures and acidities, dissociation into half molecules is complete. The frictional ratio for the half molecule was calculated to be 1.21 and 1.92 for the whole molecule. Studies on the fractions of gliadin revealed that it is not a simple homogeneous protein.

Lamm and Polson (58) introduced a refractometric method for measuring diffusion constants of proteins. Gliadin, one of the proteins studied, was found to heterogeneous, as indicated by the deviation of the diffusion curve from the theoretical. The diffusion constant was calculated to be 6.72×10^{-7}, and the molecular weight calculated from sedimentation and diffusion constants was 27,500.

The molecular weight of gliadin was calculated by Burk (13) from osmotic pressure measurements using various solvents. In alcoholic solution the molecular weight was 40,900; in 6.66M urea in 0.05M phosphate buffer, pH 7.2-7.5, 44,200; in 75% glycerol 0.02M in phosphate, 67,000; in 15% urethane 0.02M in phosphate, 75,300; and in salt free urethane, 42,000. The extent of association of gliadin, and therefore its molecular weight, was found to be dependent on the solvent used.

Oncley (76) determined the molecular weights of a number of proteins by dielectric measurements. Procedures were adapted for the measurement of both the dielectric constant and the conductance of protein solutions. The dielectric increment, i.e., the difference in the dielectric constants between that of solution and of solvent, can be interpreted in terms of the dipole moment and the size and the shape of the protein molecule. On this basis a molecular weight of 42,000 was calculated for gliadin in 56% aqueous ethanol.

The most detailed study of gluten and its fractions was made by McCalla and Gralén (63) using sedimentation and diffusion data. Under carefully controlled conditions, fractions of gluten were obtained both by dilution and by addition of magnesium sulfate. The four fractions obtained by addition of salt were characterized by diffusion and by sedimentation. A definite increase was observed in sedimentation coefficient and in molecular weight with decreasing solubility of the fractions. The molecular weight for the least soluble fraction calculated by sedimentation equilibrium was 1,750,000 and for the most soluble, 44,000. Much of this high molecular weight

fraction was recognized as aggregated. All properties showed progressive changes both within and between the arbitrarily produced fractions, providing concrete evidence that gliadin and glutenin are not distinct entities and that gluten is a protein complex having progressive and regular changes in properties with change in solubility.

The same workers (64) studied glutens from wheats of widely differing quality again by sedimentation and diffusion measurements. It was concluded that with glutens from different types of wheat, sedimentation velocity measurements are useful in determining the proportion of protein molecularly dispersed. The amount so dispersed in 8% sodium salicylate was 34 - 68% of the total. No other differences in the fundamental properties of the dispersed protein, as determined by sedimentation and diffusion methods, could be related to the differences in gluten quality.

Electrophoretic and diffusion studies were conducted by Colvin and McCalla (17) on gluten. The results of this study indicated that gluten in sodium salicylate is electrostatically homogeneous with a high mean negative valence of 34. The size and shape of the gluten particles were calculated to be prolate ellipsoids of revolution of the order of 25 Å in diameter and 400-450 Å long. It was suggested that the degree of hydration of gluten particles, on the basis of the axial and frictional ratios, is not great.

A detailed study of gliadin using electrophoretic, sedimentation and light scattering data was made by Holme and Briggs (45). Electrophoretically, gliadin was found to be heterogeneous. Using the

figures of Krejci and Svedberg for the diffusion constant and partial specific volume, a molecular weight of 24,000 was obtained. By light scattering, on the other hand, values ranging from 250,000 for crude gliadin to 26,500 for the most soluble fraction were calculated.

Jones et al. (51) determined the molecular weights of wheat gluten fractions using the approach to sedimentation equilibrium. The fractions were separated electrophoretically. The molecular weight of one of the gliadin fractions was calculated to be 47,000 and another 42,000. Glutenin was found to be heterogeneous, with a weight average molecular weight of 2 - 3 million but containing small molecules of molecular weight approximately 50,000 as well as very large ones.

The molecular weight of the glutenin fraction of wheat before and after splitting the disulfide bonds was determined by Nielsen et al. (74). Glutenin was separated by adding sodium acetate to gluten dispersed in dilute acetic acid. Disulfide bonds were oxidatively cleaved by performic acid and also reduced by reaction with sodium sulfite. Electrophoretic analysis showed that glutenin, oxidized and reduced glutenin were homogeneous in aluminum lactate buffer. The molecular weight of glutenin using the approach to sedimentation equilibrium was calculated to be about 300,000, whereas that of both oxidized and reduced glutenin was 20,000. It was suggested that glutenin is composed of basic peptide units of 20,000 which are linked via disulfide bonds to form the higher molecular weight molecules.

Taylor and Cluskey (106) determined the diffusion constants of gluten, gliadin, glutenin and glutenin purified four times. Diffusion constants determined at 1°C were 0.97 x 10^7 cm^2/sec. for gluten, 2.1 for gliadin, 0.53 for glutenin and 0.30 for 4x glutenin. Although these values are not corrected to 20°C, as is usually done, corrected values would still be far smaller than those reported in the literature. The distribution of sedimentation coefficients was also studied and it was found that for gluten and its components, there was a broad distribution. These distribution curves were corrected neither for diffusion nor for the dependence of S on concentration. Therefore, these curves cannot be truly considered as representative of gluten or of its components.

Molecular weights calculated from sedimentation and diffusion were: 100,000 for gluten, 60,000 for gliadin and 1,000,000 for glutenin. The results presented in this paper are radically different from those presented in the earlier literature and will be considered fully in the "Discussion" section.

Amino Acid Analysis

The observation that proteins were cleaved by the hydrolytic action of strong acids into relatively simple crystalline substances was made well over a century ago. Although all of the naturally occuring amino acids were isolated and characterized by 1922, it was not until 1950 that the first complete analysis for amino acids in wheat gluten was reported.

The first extensive amino acid analysis on the gliadin fraction of wheat was made by Abderhalden and Samuely (1) in 1905. Eleven amino acids were isolated accounting for 47% of the total protein. Osborne (77) published his famous monograph in which the amino acid composition of the various fractions of wheat was reported. Separations of the acids were effected by initial precipitation of glutamic acid as the hydrochloride, followed by fractional distillation of the remaining acids and precipitation of the acid or of its derivative. The individual acids were identified by analysis of carbon, hydrogen and nitrogen. Later (78), he increased the time of hydrolysis for gliadin and recovered more valine, leucine, proline and glutamic acid. The combined results are presented in Table I. The results of Abderhalden and Samuely are comparable to Osborne's results, except for proline and glutamic acid. Only 2.4% proline and 27.60% glutamic acid were recovered.

Damodaran (26) reported the amino acid composition of glutenin using a new technique introduced by Brazier (11). Separation of amino acids was effected by taking advantage of the difference in solubility of their copper salts in water and in methyl alcohol.

TABLE I

OSBORNE'S AMINO ACID ANALYSIS

	Gliadin	Glutenin
Glycine	0	0.89
Alanine	2.00	4.65
Aminovalerianic acid (Valine)	3.34	0.24
Leucine	6.62	5.95
α-Proline	13.22	4.23
Phenylalanine	2.35	1.97
Aspartic acid	0.58	0.91
Glutamic acid	43.66	23.42
Serine	0.13	0.74
Tyrosine	1.20	4.25
Cystine	0.45	0.02
Lysine	0	1.92
Histidine	0.58	1.76
Arginine	3.16	5.72
Ammonia	5.11	4.01
Tryptophan	trace	trace

(Figures reported as % of protein of theoretical nitrogen content of 17.5%)

The extraction of the amino acid-copper salts with these two solvents gave three fractions:

I. Copper salts soluble in methyl alcohol: valine and proline.

II. Copper salts insoluble in methyl alcohol, but soluble in water: glycine, alanine, aspartic acid, glutamic acid, arginine, histidine, lysine and tyrosine.

III. Copper salts insoluble in methyl alcohol and in water: leucine, phenylalanine and aspartic acid. He was unable to separate threonine, histidine, lysine, arginine, serine, cystine, methionine and isoleucine. His results are not very different from those of Osborne's.

Stockelback and Bailey (101) attempted an analysis of amino acids in gluten fractions using Brazier's method (11). Fourteen amino acids were separated and differences in composition between fractions were reported. However, as their results are totally different from any reported to date they will not be considered.

Gluten from an Italian wheat was analysed for amino acids by Padoa (79). Four amino acids, threonine, serine, methionine and isoleucine were undetected, and of the fourteen isolated, only six fall in the range of values determined by the more reliable column chromatography technique.

Using a microbiological assay method, Rice and Ramstad (84) determined the amino acid composition of gluten from wheat and carob. Only serine was undetected. Gluten was washed out from a commercial strong baker's patent flour. Their results are presented in Table II together with later determinations.

TABLE II

RECENT AMINO ACID ANALYSIS OF GLUTEN

	Rice (84)	Pence (80)	Waldschmidt-Leitz (112)	Woychik (115)
Alanine	1.9	2.2	3.9	2.6
Arginine	3.6	4.7	1.8	2.6
Aspartic acid	3.0	3.7	5.1	3.2
Ammonia		4.5	3.7	5.6
Cystine	2.4	1.9	3.6	2.3
Glutamic acid	31.3	35.5	35.7	40.8
Glycine	3.1	3.5	3.0	3.4
Histidine	2.1	2.3	4.5	2.4
Isoleucine	3.9	4.6	7.0	4.4
Leucine	6.1	7.6	6.3	7.4
Lysine	1.5	1.8	0	1.3
Methionine	1.4	1.9	0.8	1.3
Phenylalanine	4.8	5.4	3.1	5.4
Proline	11.7	12.7	9.3	15.0
Serine		4.7	3.7	5.7
Threonine	2.3	2.6	2.9	2.7
Tryptophan	1.0	1.1		1.1
Tyrosine	3.7	3.1	5.4	4.2
Valine	3.8	4.7	5.5	4.5

All figures reported as % of protein of theoretical N content of 17.5%.

Immediately after this publication, Pence et al. (80) reported the first complete amino acid analysis of gluten. Flour from seventeen different wheats, including hard, soft and durum wheats, were used. Microbiological assay method was applied for all but two amino acids. Tryptophan was determined colorimetrically with p-dimethylaminobenzaldehyde, hydrochloric acid and sodium nitrite. Cystine was also determined colorimetrically with p-aminodimethylaniline, ferric iron and zinc.

In the same year another report was published from Rutger's University (9) in which the amino acids of various protein food sources, including wheat gluten, were determined microbiologically. The results are very similar to those of Pence (80) except for three amino acids. Higher values were found for cystine (2.6%), proline (15.4%) and glutamic acid (38.3%).

The greatest advance made to date in the quantitative determination of amino acids has been the application of chromatography to protein hydrolysates. Chromatography on starch and on paper was introduced by Martin and coworkers (18, 59, 104) and later improved by Stein and Moore (71, 72, 99). The first report on the amino acid composition of gluten using this technique was made by Miller et al (70). Three methods, microbiological, chromatographic and chemical, were used to determine eleven amino acids. A starch column prepared according to Stein and Moore (99) was used to isolate isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, and valine. Arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan and valine were de-

termined microbiologically. Histidine was determined colorimetrically as well, with p-diazobenzene sulfonic acid. Their results are comparable to those of Pence (80).

Partition paper chromatography was used by Reznichenko et al. (83) in determining the amino acids of wheat and rye gliadins. Fifteen amino acids were identified and quantitative calculations were made for seven: glutamic acid 44.2%, proline 12.8%, leucine 6.2%, lysine 1.1%, valine 4.2%, phenylalanine 3.7% and threonine 5.0%.

Waldschmidt-Leitz and Mindemann (112) determined the amino acids of the glutenin fraction from barley, wheat and rye by chromatography on starch following the method of Stein and Moore (99). Their results are presented in Table II. Strusi (102) attempted to explain the difference in the characteristics of hard and durum wheats by analyses of amino acids by paper chromatography. However, he did not make quantitative calculations, but reported that the same amino acids were present in both, probably in a different sequence, however. Woychik et al. (115) reported the amino acid composition of gluten and the various fractions obtained by electrophoretic separations. An ion exchange column as described by Moore, Spackman and Stein (72) was used. Cystine was determined as cysteic acid by oxidation with performic acid.

Meredith et al. (68) fractionated gluten by extracting with a methanol-chloroform mixture, the fractions roughly corresponding to gliadin and glutenin. Amino acid analyses of the fractions show a higher content of glutamic acid and a lower content of proline in the 'glutenin' fraction. This is surprising, since from the time

of Osborne, it has always been found that the more soluble gliadin fraction is higher in glutamic acid and lower in proline. This difference can well be due to the method of fractionation.

Between 1920 and 1950 a number of reports were published in which a specific method for a certain amino acid was used to determine its content in a variety of proteins, among them gliadin and glutenin of wheat. The tryptophan content was determined colorimetrically to be 1.05% (61), 1.09% (47) for gliadin; 1.72% (47), 1.80% (61) for glutenin; 0.66% (87) microbiologically and 0.65% (7) by ultraviolet spectrophotometry for gliadin. For whole gluten, by a microbiological method the tryptophan content was found to be 0.93% (36). The cystine content determined colorimetrically was reported to be 1.42% (47), 2.19% (107) for gliadin and 1.56% (47) for glutenin. The glutamic acid content by a number of methods was found to be 43.0% (48), 46.92% (4), 39.0% (5), 43.0% (49), 45.7% (75) in gliadin and 25.7% (48), 35.9% (75) in glutenin. By precipitating aspartic acid with a metal 0.8% (48), 1.40% (4), 0.5% (49) were found in gliadin and 2.0% (48) in glutenin. The tyrosine content of gliadin was estimated to be 3.14% (87) by a microbiological assay method, 2.35% (37) by precipitation with mercury, and 3.4% (7) by ultraviolet spectrophotometry. Again by precipitation with a heavy metal, 9.86% (98), 10.34% (108) proline was found in gliadin and 5.98% (98) in glutenin. The histidine content was reported to be 2.1% (37) by silver precipitation for gliadin and 1.79% (111) determined colorimetrically for glutenin. By a microbiological method 6.5% leucine (87, 10) was found in gliadin, and 1.56% (82) methionine, 3.5% alanine

(90), 3.5% glycine (67) in whole gluten.

Combining all these results up to 1956, an average value for the amino acid composition of wheat gluten is given by Block and Weiss (9) to be:

alanine	2.3	histidine	2.1	serine	4.7
arginine	3.9	isoleucine	4.6	threonine	2.7
aspartic acid	5.3	leucine	7.4	tryptophan	1.0
cystine	2.2	lysine	1.8	tyrosine	3.4
glutamic acid	33.6	methionine	1.8	valine	4.7
glycine	4.4	phenylalamine	5.6	ammonia	4.3
		proline	12.1		

(as % of protein of theoretical N content of 17.5%)

These figures of Block and Weiss are based on determinations made by the older methods - microbiological assay method and chemical - and therefore, should not be accepted as the ultimate authority on amino acid composition, but are invaluable as a guide. Results obtained by chromatography are considered more reliable but until complete amino acid sequences of the various components of gluten are determined, a true amino acid composition cannot be accepted.

The sulfhydryl-disulfide system of gluten

A great deal of attention has been focussed on the sulfhydryl-disulfide system of proteins. Numerous methods for detecting the number of free sulfhydryl groups and the total sulfhydryl-disulfide content have been applied in attempting to elucidate the role of this system in the reactions and the structure of proteins.

One of the earliest studies in which the sulfhydryl-disulfide system was implicated in the behavior of dough was made by Sullivan et al. (103). The effect of oxidizing and reducing agents on dough was studied and from their results, it was suggested that the disulfide linkage was the reactive group which caused the proncunced changes in the physical properties of dough.

The effect of reducing agents on gluten was studied by Hlynka (44) and by Pence and Olcott (81). Bisulfite, acetaldehyde and similar reagents were incorporated into the dough and gluten washed out by Hlynka. The changes in the gluten properties were believed to be due to the rupture of the disulfide bond or possibly the cross-linkages formed by carbonyl compounds. Pence and Olcott added reducing agents to gluten dispersed in dilute acetic acid. Relative viscosity and cystine content determinations on treated and untreated gluten led them to conclude that the sulfhydryl-disulfide system is directly involved in the changes brought about by reducing agents on gluten.

Gluten was reduced with thioglycolic acid and the sulfhydryl content determined by ferricyanide oxidation in the presence of urea by DeDeken and DeDeken-Grenson (28). Their value of 0.94 μM SH/mg protein N, representing the total sulfhydryl-disulfide when

converted to total cystine agrees with that obtained by Pence et al. (80) in their amino acid analysis.

A polarographic method was used by de Lange and Hintzer (57) for determining the sulfhydryl content of wheat. Values ranging from 0.28 to 0.61% of the gluten protein were found for thirteen varieties of wheat. Total cystine content was found to range from 1.84 to 2.78% for gluten. It was suggested that the ratio of sulfhydryl to disulfide may be an important factor in gluten properties.

Studies on the sulfhydryl-disulfide groups of other proteins, e.g., serum and plasma albumins, led investigators to postulate an interchange reaction (46):

$$R_1SH + R_2S\text{-}SR_3 \longrightarrow R_1S\text{-}SR_2 + R_3SH$$
$$\text{or} \quad R_1S\text{-}SR_3 + R_2SH$$

This type of interchange reaction is believed by many cereal chemists to be operative in dough.

Goldstein (34) found that the specific blocking of the sulfhydryl groups of wheat gluten with p-chloromercuribenzoate resulted in an improvement of the mechanical properties of dough. This was interpreted as indicating an exchange reaction being prevented, resulting in improvement of dough. Deterioration of mechanical properties of dough is believed to be caused by an exchange reaction. After addition of a sulfhydryl-blocking reagent, oxidizing improvers such as bromate, had no effect. It was suggested that the sulfhydryl groups were involved in the chemical action of flour improvers.

Amperometric titration at a rotating platinum electrode with mercuric chloride and silver nitrate was used by Kong et al. (55) for the estimation of sulfhydryl groups in wheat flour. Under

various conditions, values ranging from 0.17 to 0.31 μeq. SH/g flour was obtained. Sokol et al. (94), under conditions believed to minimize sulfhydryl losses prior to actual titration, reported a higher range of values, 0.66 - 1.30 μeq/g. flour. The conditions were: extraction of flour at 3°C, use of 8M urea and ethylenediaminetetraacetate, and titration with silver nitrate at pH 7.5.

Schaeffer et al. (92) attempted to titrate sulfhydryl groups of gluten and other flour components with iodine. Actual sulfhydryl content was not reported, only the equivalents of iodine reacted. Since iodine is readily reduced by a variety of compounds, the amount reduced cannot be directly related to the sulfhydryl content. This was found to be the case in the reaction of iodine with β-lactoglobulin and ovalbumin where two equivalents of iodine more than the theoretical amount required for the oxidation of sulfhydryl groups reacted. (25)

The sulfhydryl and disulfide content of low protein flour and flour extracts were measured by amperometric titration of excess methyl mercuric iodide by Frater et al. (32). Flour in the presence of urea was found to contain 450 μM SH/300 g flour and 184 μM without urea. Rheological properties of dough were studied with the Brabender farinograph and extensograph after treatment with iodate, bromate, N-ethylmaleimide and cysteine. On the basis of their results, it was suggested that the rheological properties are directly related to the number of disulfide bonds and the rate at which sulfhydryl-disulfide interchange reaction can take place.

Matsumoto and Hlynka (60) determined the sulfhydryl content of flour and acid extracts of flour by amperometric titration with sil-

ver nitrate in the presence of 4.8 M urea. Extractions and titrations were carried out under an atmosphere of nitrogen. The total sulfhydryl content found for flour was 56.2 μM/100 g. flour, a value appreciably lower than that reported by Frater et al. (32).

The kinetics of the bromate reaction in dough when seventeen different chemicals were added, was studied by Bushuk and Hlynka (15). Among the seventeen chemicals were sulfhydryl-blocking reagents N-ethylmaleimide and iodoacetamide, as well as metal ions Cu (II), Hg (II), Ag and Se which are known to react with sulfhydryl groups. The decrease in the rate of bromate reaction in the presence of these ions and reagents was interpreted as indicating that the sulfhydryl group is the principal component involved in the bromate reaction in dough.

Bushuk (14), by back titration of iodate amperometrically, determined the accessible sulfhydryl groups in dough. Iodate has been found to react immediately with some component(s) of flour. The reactive group in flour has been assumed to be the sulfhydryl groups. When N-ethylmaleimide was incorporated into the dough prior to the addition of iodate, all the added iodate was recovered. This was concluded to be a specific reaction of iodate with the sulfhydryl groups. On this basis, the number of accessible sulfhydryl groups varied from 4.0 to 7.4 μeq/g. protein, depending on the protein content of the flour. Durum wheat was found to contain 7.5 μeq/g. protein.

In many of the studies involving sulfhydryl-disulfide groups of proteins, urea is commonly used to unfold the protein and to expose masked sulfhydryl groups. It has been pointed out by Stein (100) that many of the so-called denaturing effects of urea may be

attributed to the small amount of cyanate ion which is always present with urea. It is now known that 8M urea solution is about 0.02 M in cyanate at equilibrium. Depending on the purity of urea, or the temperature of the medium, enough cyanate may be present to be damaging to protein containing amino, sulfhydryl or reactive disulfide groups. Disulfide bonds in the presence of cyanate may be hydrolysed according to the reaction

$$R_1SSR_2 \overset{HOH}{\rightleftharpoons} R_1SH + HOSR_2$$

Cyanate being a sulfhydryl reagent will react with the mercaptan and cause the equilibrium to be displaced toward the right.

A similar effect of N-ethylmaleimide has been found by Spackman et al. (96). When oxidized glutathione and cystine were incubated at 40°C, pH 6.7 for 30 hours with N-ethylmaleimide, 27% cystine and 8% glutathione were lost. Together with guanidine hydrochloride, N-ethylmaleimide caused a loss of 23% glutathione and 44% cystine. The reaction of N-ethylmaleimide is similar to that of cyanate in that the equilibrium reaction shown above, is displaced toward the right. If the sulfhydryl content is to be determined by measuring the decrease in the concentration of N-ethylmaleimide, this must be taken into consideration.

MATERIALS AND METHODS

Three flours from Red Bobs (hard red spring), Lemhi (soft) and Mindum (durum), milled on an experimental Buhler Mill and extracted with ether, were used for the experiments. The protein and moisture content of each of the flours were:

	Protein (Nx5.7) dry basis	Moisture
Red Bobs	16.2%	10.9%
Lemhi	11.4	12.5
Mindum	13.9	10.0

Glutens were washed out by hand using phosphate buffer, pH 6.8, according to the method of Dill and Alsberg (30). Excessive mechanical manipulations were avoided in gluten washing and in dispersion as the properties of gluten are relatively easily altered, as found by McCalla and Gralen (63). Glutens from 10 g. flour were placed in glass-stoppered flasks with 50 ml. of 8% sodium salicylate and left at room temperature with occasional stirring. After all gluten had dispersed, the dispersions were centrifuged at 11,000 g. for 20 minutes in an angle centrifuge at 3°C to remove the starch. The supernatants were dialysed against 8% sodium salicylate at 3°C for 24 hours for all experiments.

Some preparations were made under an atmosphere of nitrogen as a precaution against oxidation of sulfhydryl groups by atmospheric oxygen. Flour samples were placed in a desiccator, evacuated, then equilibrated with nitrogen and left overnight. Flour samples were then transferred to a plastic bag which was arranged so that it could be evacuated and filled with nitrogen. All solutions used were bubbled with nitrogen for 20 minutes to remove any dissolved

oxygen. Doughs were mixed, gluten washed out and dispersions in sodium salicylate were all done under nitrogen.

All chemicals used were reagent grade, and when obtainable only in technical grade, were purified either by recrystallization or distillation. N-ethylmaleimide obtained from Nutritional Biochemicals was recrystallized twice from xylene, dried and kept at 3°C. Cysteine hydrochloride was recrystallized twice from 6 N hydrochloric acid, dried in vacuo and found to be chromatographically pure. Thioglycollic acid was purified by vacuum distillation. Methyl mercuric nitrate was prepared by reacting stoichiometric amounts of methylmercuric hydroxide and silver nitrate. The precipitated silver oxide was separated by filtration and methylmercuric nitrate was purified by recrystallization from methyl alcohol. A dilute solution of methylmercuric nitrate was standardized with thioglycolic acid using nitroprusside as indicator. The p-chloromercuricbenzoic acid used was obtained from Mann Research Laboratories.

Fractionation of gluten was effected by dilution, adding equal volumes of distilled water and gluten dispersed in 8% sodium salicylate. This mixture was stored overnight at 3°C then centrifuged at 11,000 g. for 20 minutes to remove the precipitated protein. The supernatant was dialysed against 8% sodium salicylate for 24 hours at 3°C. The reproducibility of the fraction was tested and it was found that, under constant conditions, the fraction on the basis of proportion of total nitrogen was reproducible.

Viscometric studies were done with the Cannon-Ubbelohde dilution viscometer at 20 $\pm$ .05°C. All solutions were filtered through

a sintered glass filter of medium porosity. Intrinsic viscosities were calculated according to the following derivation.

If the viscosity of the solvent is η_o , the viscosity of solution η , of concentration c, can be expressed as a power series

$$\frac{\eta}{\eta_o} = 1 + Ac + Bc^2 + \ldots\ldots \quad (1)$$

η/η_o is the relative viscosity. Frequently this equation is written as

$$\frac{\eta_{sp}}{c} = A + Bc + \ldots\ldots \quad (2)$$

where $\eta_{sp} = \eta/\eta_o - 1$ and is known as the reduced viscosity. At infinite dilution, when Bc and the following terms are negligible, the intrinsic viscosity $[\eta]$ is defined as

$$\lim_{c \to 0} \left(\frac{\eta_{sp}}{c} \right) = A = [\eta] \quad (3)$$

Using equation (1) by plotting η/η_o versus c, a straight line is obtained with intercept = 1, and slope = $[\eta]$. The slopes of the lines were calculated by the method of least squares.

Sedimentation velocity runs were made on the Spinco Model E ultracentrifuge equipped with a phase plate and an electronic temperature control unit. Initially, runs were made at 59,780 rpm but later due to the age of the rotor, the speed was reduced to 52,640 rpm. Sedimentation coefficients were calculated using the equation

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt}$$

which upon integration gives

$$s = \frac{2.303 \log (x_2 / x_1)}{\omega^2 (t_2 - t_1)}$$

where x_2 = distance in cm of peak from centre of rotation at time t_2

x_1 = distance in cm of peak from the centre of rotation at time t_1

w = angular velocity of rotor in radians/sec.

Since sedimentation coefficients are generally reported as $s_{20,w}$, the value the material would have in a solvent with the density and viscosity of water at 20°C, corrections of the observed sedimentation coefficients were made to the standard state according to the equation

$$s_{20,w} = s_{obs} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_o}\right) \frac{(1 - \bar{v}\rho_{20,w})}{(1 - \bar{v}\rho_t)}$$

η_t/η_{20} = viscosity of water at t°C relative to that at 20°C

η/η_o = relative viscosity of solvent to that of water

$\rho_{20,w}$ = density of water at 20°C

ρ_t = density of solvent at t°C

$\bar{v}$ = partial specific volume

All density measurements were made with either a 50 ml. or 10 ml. pycnometer at 20 $\pm$ .05°C. The exact volume of the pycnometers used was determined by using distilled water.

Molecular weights were determined by the approach to sedimentation equilibrium method using the model E ultracentrifuge at 12,590 and 15,225 rpm. The outline of the procedure given by Schachman (91) was followed.

The equation used for molecular weight calculations was

$$M = \frac{RT}{(1-\bar{v}\rho)\omega^2}\,\frac{(dc/dx)_m}{x_m c_m} = \frac{RT}{(1-\bar{v}\rho)\omega^2}\,\frac{(dc/dx)_b}{x_b c_b}$$

M = molecular weight of protein

$\bar{v}$ = partial specific volume of the protein

R = gas constant, 8.314×10^7 ergs/mole/degree

T = absolute temperature

ρ = density of solution

ω = angular velocity in radians/second

c_m = concentration at position x_m

$(dc/dx)_m$ = concentration gradient at position x

x_m = distance in cm. from axis of rotation to the meniscus.

The corresponding quantities with the subscript b refer to the bottom of the cell. The values of c_m and c_b are calculated by the following equations

$$c_m = c_o - \frac{1}{x_m^2}\int_{x_m}^{X} x^2\,(dc/dx)\,dx$$

$$c_b = c_o + \frac{1}{x_b^2}\int_{X}^{x_b} x^2\,(dc/dx)\,dx$$

c_o = initial concentration

X = position in the plateau region, i.e. $dc/dx = 0$

A conventional 12-mm. cell with a 4° sector centerpiece was used for these runs. A few drops of carbon tetrachloride were added to the cell to eliminate convective disturbances and to facilitate the measurements of the patterns at the cell bottom. Another run of the same solution was made on the synthetic boundary cell to calculate the value of c_o. These patterns were enlarged with a slide projector and all measurements were made from the magnified diagrams. The area for the determination of c_o was measured with a planimeter.

A few diffusion experiments were carried out on the Klett-electrophoresis apparatus. The water bath was controlled at 20 $\pm$ .05°C. The gluten fraction in 8% sodium salicylate was dialysed for 24 hours, placed in a Tiselius cell and the solvent was carefully layered on top with a syringe. The boundary was sharpened by inserting a finely drawn glass tube at the boundary and withdrawing the solution by gentle suction. Photographs were taken 1, 3, 6, 12, 24 and 36 hours after formation of the boundary. Diffusion coefficients were calculated by the method of maximum ordinate.

The diffusion curve is described by the equation

$$\frac{dc}{dx} = \frac{c}{2\sqrt{\pi D t}} e^{-x^2/4Dt}$$

dc/dx = concentration gradient

c = concentration

t = time in seconds

D = diffusion constant

dc/dx is directly related to dn/dx, the refractive index gradient.

At maximum ordinate, i.e. at x = o

$$\left(\frac{dn}{dx}\right)_{x=o} = Y_{max} = \frac{c}{2\sqrt{\pi D t}}$$

The concentration is replaced by the area and the rearranged equation takes the following form:

$$t = \frac{A^2}{4\pi D\, Y_{max}^2}$$

t is plotted against $1/Y_{max}^2$; a straight line is obtained with slope $= A^2/4\pi D$

The slope is calculated by the method of least squares.

Combination of diffusion and sedimentation constants can be used to calculate molecular weights from the equation:

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

The symbols have the same meaning as in previous equations.

Sedimentation velocity diagrams can be converted to distribution of sedimentation coefficients (12, 66 and references cited therein). If the diffusion coefficient is negligible and the sedimentation coefficient is independent of concentration, the refractive index gradient curve can be converted directly into a distribution of sedimentation coefficients by the expression:

$$g(s) = \left(\frac{dc}{dx}\right) \frac{\omega^2 t x^3}{c_o x_o^2}$$

where $g(s)$ = distribution function

ω = angular velocity in radians/sec.

t = time in seconds from the start of sedimentation

x = distance in cm. of a point in the boundary from the axis of rotation

x_o = distance in cm. of the meniscus from the axis of rotation

c_o = total concentration of the solution

The entire distribution curve was obtained by calculating the apparent distribution functions $g^*(s)$ from each of 10 to 20 points describing the refractive index gradient curve and plotting against corresponding sedimentation coefficients. The sedimentation coefficients, calculated using the equation,

$$s = \frac{1}{\omega^2 t} \ln \frac{x}{x_o}$$

were corrected to the standard value, $s_{20,w}$. When diffusion is not negligible, the diffusional effects were eliminated by extrapolating to infinite time. This was done by calculating the apparent distribution functions for sedimentation diagrams at increasing times. From these curves, discrete values of s (i.e., 1, 1.5, 2, etc.) were taken and plotted as $g^*(s)$ versus 1/xt. A graphical extrapolation was then made to 1/xt = 0 to yield values of the apparent distribution, corrected for diffusion.

Since s values for gluten and the fraction in 8% sodium salicylate were found to be essentially independent of concentration, corrections for s = f(c) were not made.

Sedimentation velocity runs were carried out at 52,640 rpm at 20°C. A conventional 12mm 4° sector cell was used. Base line correction was made by determining the sedimentation pattern of the solvent separately. Both crude gluten and the fraction from the three wheats in 8% sodium salicylate were studied. One run was made with bovine serum albumin in 0.01 M phosphate buffer to determine the shape and the breadth of the distribution curve for a fairly pure protein for comparison.

Several methods for the determination of free sulfhydryl groups were tried. A spectrophotometric method with ferricyanide (52), and titration with methyl mercuric nitrate (2) proved unsatisfactory as gluten precipitated under the conditions specified and a suitable buffer system could not be found. A colorimetric method using Folin's reagent (54) was also found to be unsatisfactory.

The procedure finally adopted was to determine the total sulfhydryl - disulfide content as cysteic acid by performic acid oxidation. If the free sulfhydryl groups are first blocked with reagents such as p-chloromercuribenzoate, N-ethylmaleimide or methyl mercuric nitrate, then oxidized with performic acid, only the disulfide groups are oxidized to sulfonic acid, while the blocked sulfhydryl groups are oxidized to sulfones. The sulfhydryl content would then be obtained by difference between cysteic acid of treated and untreated samples. Doughs were mixed and the glutens washed out under nitrogen when a sulfhydryl reagent was used. To gluten from 10 g. of flour, 100 ml. of performic acid was added and left overnight at 3 °C. The dispersion was then centrifuged at 11,000 g. The volume of the dispersion was reduced to 25 ml. and an aliquot was hydrolysed. Cysteic acid was determined on the Spinco amino acid analyser. Performic acid was prepared by mixing 85% formic acid with 0.115 vol. of 30% hydrogen peroxide as described by Woodin (113).

Gluten samples were hydrolysed by refluxing with constant boiling hydrochloric acid for 24 hours. Quadruplicate runs were made on crude gluten and on the fraction soluble in 4% sodium salicylate for each of the three types of wheat. In one case, gluten dispersed in salicylate was dialysed against water and the precipitated gluten

freeze-dried. Freeze-dried samples were then hydrolysed. In others, known volumes of gluten dispersions were refluxed with acid.

Hydrolysates were filtered, then evaporated to dryness on a rotary evaporator. A water bath not exceeding 50 °C was used to heat the solution during evaporation. The dried hydrolysate was taken up in distilled water and evaporated to dryness again. Finally, citrate buffer, pH 2.2 was added to the dried samples and when necessary the pH was adjusted to 2.2 with sodium hydroxide.

Amino acid composition was determined on the Spinco amino acid analyser.

Tryptophan was determined colorimetrically following the method described by Fischl (31). To 0.5 ml. of an unhydrolysed gluten sample, 2 ml. of glacial acetic acid, 1 ml. of concentrated sulfuric acid, 1 drop of 3% hydrogen peroxide and 1 drop of 5% thioglycolic acid in glacial acetic acid were added. After 15 minutes the absorption was measured at 550 mμ in the Beckman DK 1 spectrophotometer. A calibration curve was constructed with known quantities of tryptophan.

Total nitrogen content was determined by the micro-Kjeldahl method using mercuric sulfate as catalyst. Ammonia was collected in 4% boric acid and titrated with standardized hydrochloric acid using bromcresol green indicator.

RESULTS

As differences in the physicochemical properties of glutens from the three types of wheat might be better detected in a given fraction, gluten was fractionated on the basis of solubility in sodium salicylate. It was shown by McCalla and Rose (62) that gluten can be separated into numerous fractions, none representing definite protein entities. Initially, fractionation by addition of magnesium sulfate was tried but redispersion of precipitated gluten took $1\frac{1}{2}$ to 2 days. Therefore, fractionation by dilution was used.

Results of the fractionation of gluten from the three wheats are presented in Table III

TABLE III

FRACTIONATION OF GLUTEN

	Total crude gluten from 10 g. flour dispersed in 8% sodium salicylate	Fraction soluble in 4% salicylate % of crude gluten
Red Bobs	1.035 g.	70.5%
Lemhi	0.952	63.5
Mindum	1.050	74.8

(Average of eight fractionations)

This fraction soluble in 4% sodium salicylate is slightly greater than that reported by Spencer and McCalla (97). They found that for gluten from Reward wheat, the amount dispersed in a given salicylate concentration was dependent on the purity of the salicylate, the time of dispersion, and whether the preparation was made by dilution of concentrated gluten dispersion or dispersed by repeated extraction. With C.P. sodium salicylate the amount of gluten in 4% by the dilution method was approximately 62%. The difference of 8% (for Red Bobs) might be attributed to incomplete dispersion of the original gluten due to insufficient dispersion time in the present study. However, since these fractions, prepared by dilution methods, do not represent true protein entities, the only consideration of importance is the reproducibility of the fraction.

Viscosity Studies

The viscosity values of crude gluten dispersions in several solvents were measured. Aluminum lactate, prepared according to Jones et al. (50), and aluminum glycolate, similarly prepared, were used for a few runs. Since it is well known that aluminum ions form a variety of complex ions, there is a strong possibility that aluminum-protein complexes may be formed. Thus, aluminum solvents were deemed unsatisfactory due to this possibility.

Detailed viscosity studies of gluten dispersed in 0.1 M lactic acid containing 0.01 M sodium chloride were made. In one series, gluten from each of the three wheat flours was washed and dispersed under an atmosphere of nitrogen. In another, gluten dispersions were made without exclusion of atmospheric oxygen, i.e., in air.

Figure 1 A, B and C show the effect of atmospheric oxygen on the relative viscosity of the three glutens. In all cases the relative viscosity of the dispersions prepared under nitrogen was higher than that prepared in air.

Both crude gluten and the fraction soluble in 4% sodium salicylate were studied viscometrically in 8% sodium salicylate. The fraction was prepared both under nitrogen and in air, while crude gluten was dispersed only in air. The results are presented in Figure 2. Again, higher values of relative viscosities were obtained for dispersions prepared under nitrogen.

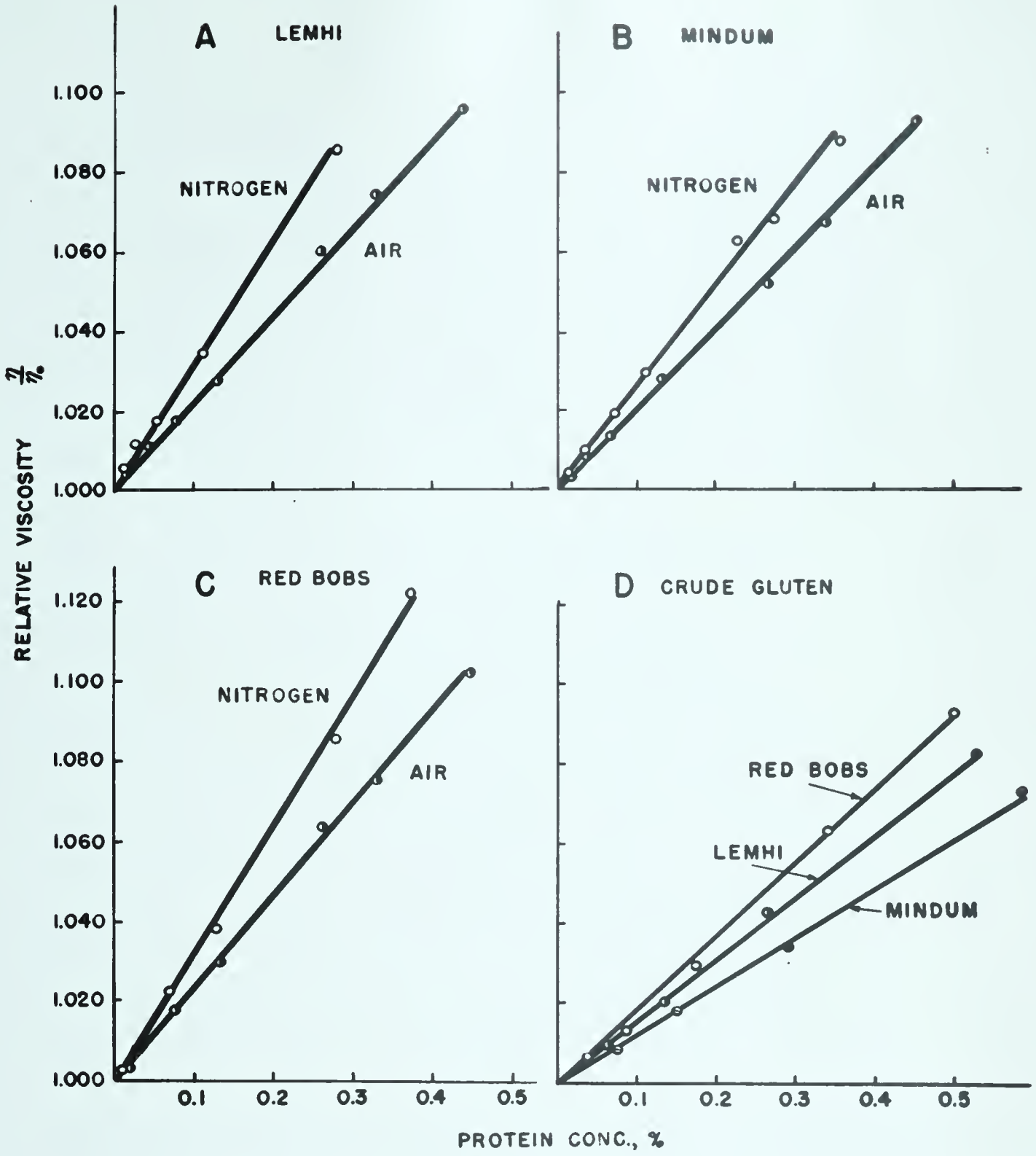


Figure 1

Effect of atmospheric oxygen on gluten from A - Lemhi, B - Mindum, C - Red Bobs. D - effect of sulfite on gluten. Dispersions in 0.1*N* lactic acid + 0.01*N* NaCl.

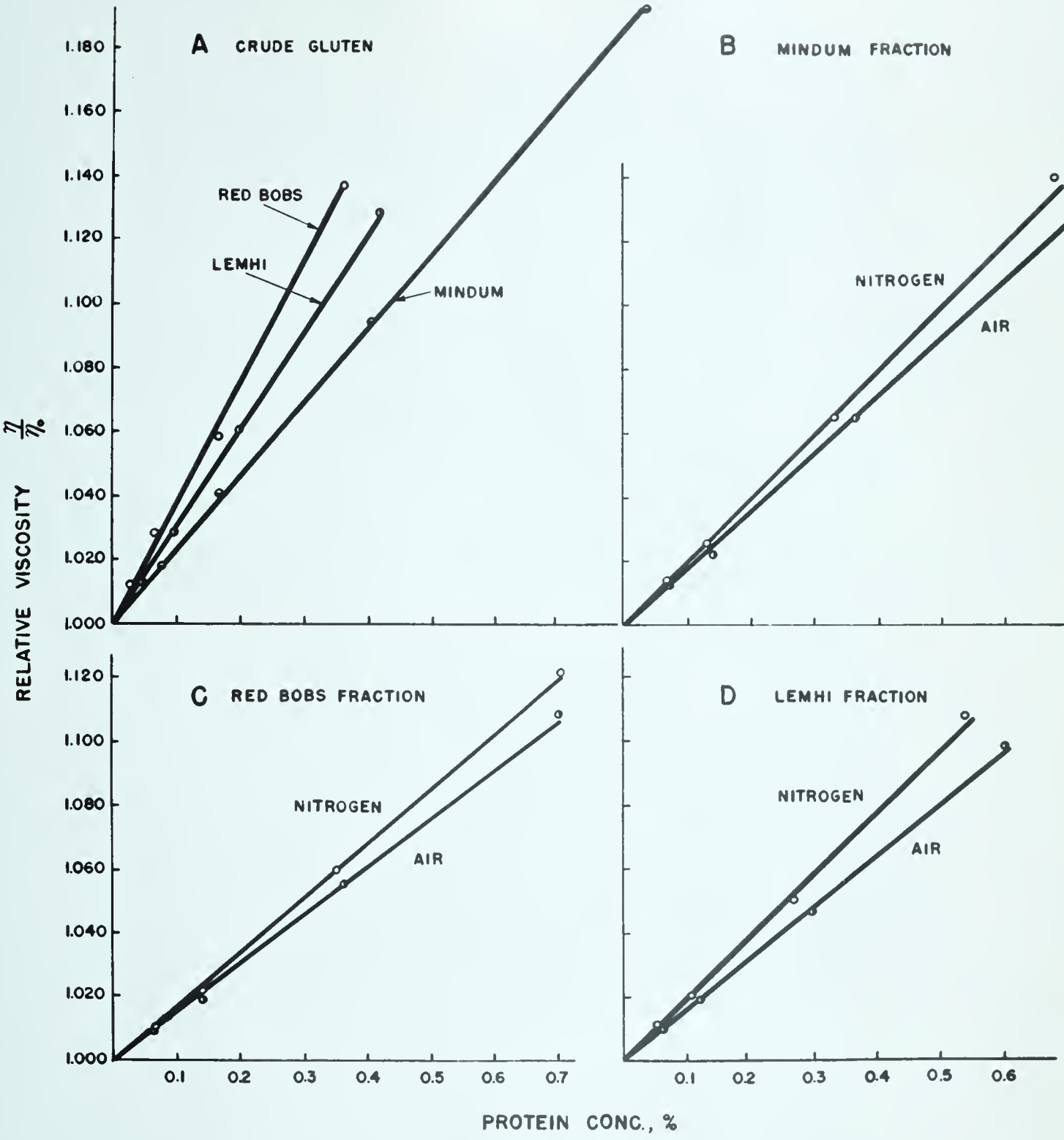


Figure 2

Effect of atmospheric oxygen on gluten fraction from B - Mindum, C - Red Bobs, D - Lemhi. A- crude gluten dispersed in air. Solvent: 8% sodium salicylate.

Intrinsic viscosities, calculated according to the method described in the previous section, are presented below.

TABLE IV

Intrinsic viscosities, dl/g, of gluten and the fraction under different conditions

	Crude gluten			Fraction	
Solvent:	8% Na Sal.	0.1 Lactic acid + 0.01 NaCl		Na Sal.	
Dispersion prepared in:	air	air	nitrogen	air	nitrogen
Red Bobs	0.37	0.24	0.33	0.15	0.18
Lemhi	0.31	0.23	0.30	0.16	0.20
Mindum	0.23	0.22	0.25	0.19	0.22

Although Harris and coworkers (33,41,42) did not calculate intrinsic viscosities and reported only absolute viscosity values, they found a similar trend in that the highest value was found for gluten from hard wheat dispersed in 10% salicylate and lowest for gluten from durum wheat, while that of gluten from soft wheat was intermediate. Intrinsic viscosities reported by Cluskey et al. (19) for gluten from hard wheat dispersed in aluminum lactate were 0.35 dl/g (mean of 13 varieties) and 0.31 dl/g for soft wheat (mean of 11 varieties). This difference was found to be statistically significant. A slightly higher value for gluten in aluminum lactate was reported by Nielsen et al. (74). They determined the intrinsic

viscosity of gluten to be 0.45 dl/g, of glutenin 0.68 dl/g and of gliadin 0.15 dl/g. Thus the results obtained in this study are comparable to those of other workers.

The trend is reversed for the fractions in that gluten from durum wheat exhibits the highest intrinsic viscosity and that from hard wheat the lowest. Although the differences are small, this trend appears in dispersions prepared both under nitrogen and in air.

The effect of sodium sulfite on the viscometric behavior of gluten dispersed in lactic acid-sodium chloride was also studied. Results are presented in Figure 1 D. Intrinsic viscosities were found to be appreciably lowered by the addition of sulfite.

Red Bobs	0.18 dl/g
Lemhi	0.16
Mindum	0.12

A similar lowering of intrinsic viscosity upon addition of sulfite was reported by Udy (109).

Sedimentation Velocity Studies

Generally, sedimentation coefficients of proteins are dependent on the concentration, increasing with decreasing concentration. To determine the concentration dependence for gluten proteins, runs were made at various concentrations in the two solvents used in viscosity studies. Both crude gluten and the fraction from Red Bobs flour were found to give sedimentation coefficients essentially independent of concentration in 8% sodium salicylate. This is concordant with the results of McCalla and Gralen (64). However, crude gluten dispersed in lactic acid-sodium chloride solution exhibits a definite concentration dependence as shown in Figure 3. Thus, sedimentation coefficients

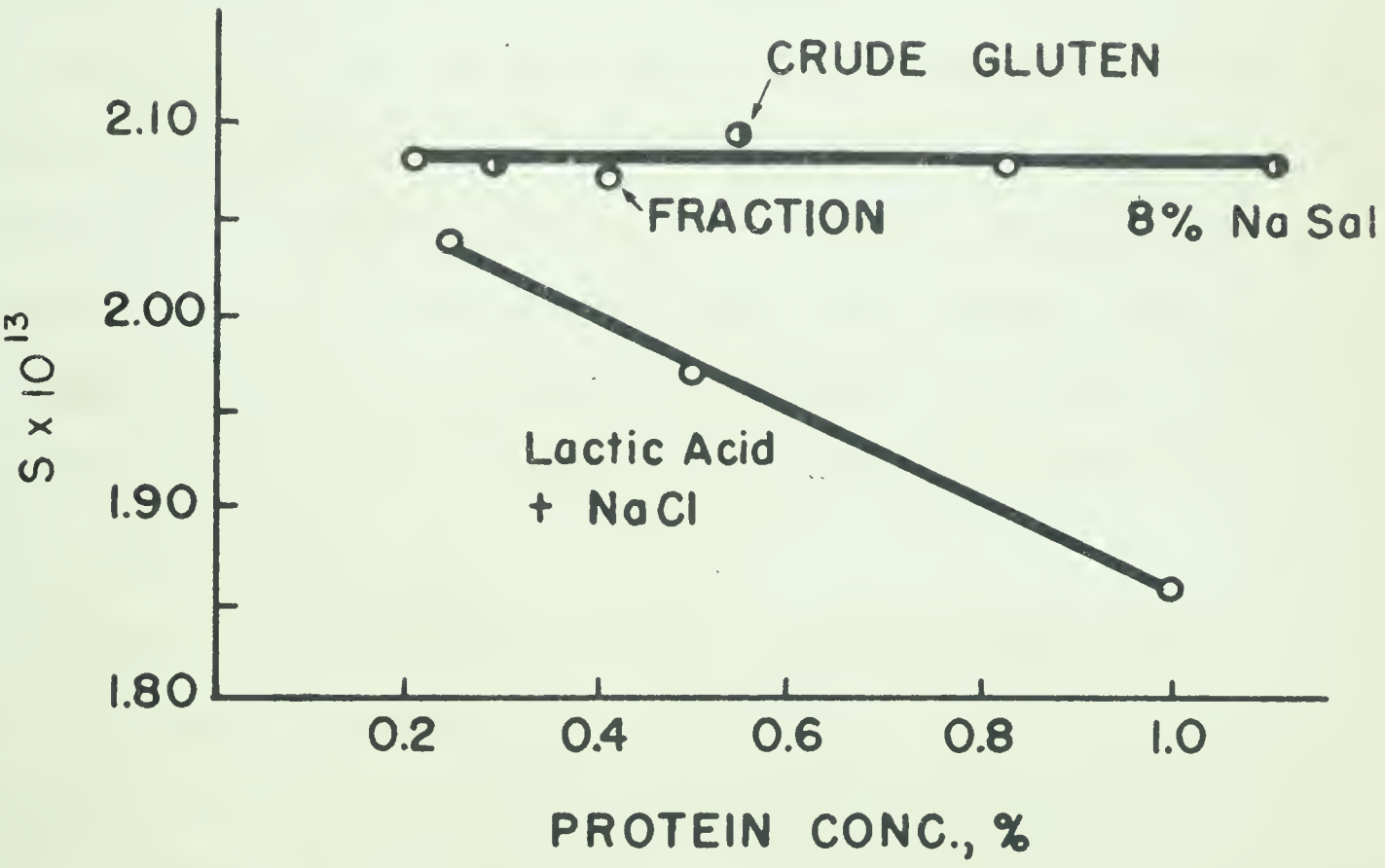


Figure 3

Effect of concentration on the sedimentation coefficient.

for gluten dispersions in solvents other than sodium salicylate must be extrapolated to zero concentration to obtain the intrinsic sedimentation coefficient in order to make comparisons.

Sedimentation diagrams of gluten from Red Bobs flour dispersed in 8% sodium salicylate, 0.1 M lactic acid - 0.01 M sodium chloride, 0.02 M aluminum lactate and 0.04 M aluminum glycolate are presented in Figure 4. Only one peak is formed in each of the four solvents. The only difference in the shape of the diagram occurs in the base line of that dispersed in 8% sodium salicylate. This can be attributed to the high concentration of salicylate, a condition that has been shown to yield poor base lines. Sedimentation coefficients, when extrapolated to zero concentration, are found to be approximately the same in all four solvents, indicating that there is essentially no difference in the hydrodynamic unit. This is in agreement with the results of McCalla and Verma (65) who found no basic differences in the sedimentation diagrams of gluten dispersed in 8% sodium salicylate, 8% sodium salicylate plus bisulfite, aluminum lactate and Swan's reagent (cupric ammonium sulfite).

Parallel to the viscometric studies, sedimentation velocity runs were made on the dispersions used in viscometry. Crude gluten dispersions in lactic acid-sodium chloride mixture from the three wheats, prepared under nitrogen and in air were studied. The diagrams for three wheat glutens, whether prepared in nitrogen or air, were all almost identical. Those prepared in air are presented in Figure 5 A, B, and C. Sedimentation coefficients for the six preparations are presented below. These values are corrected to zero

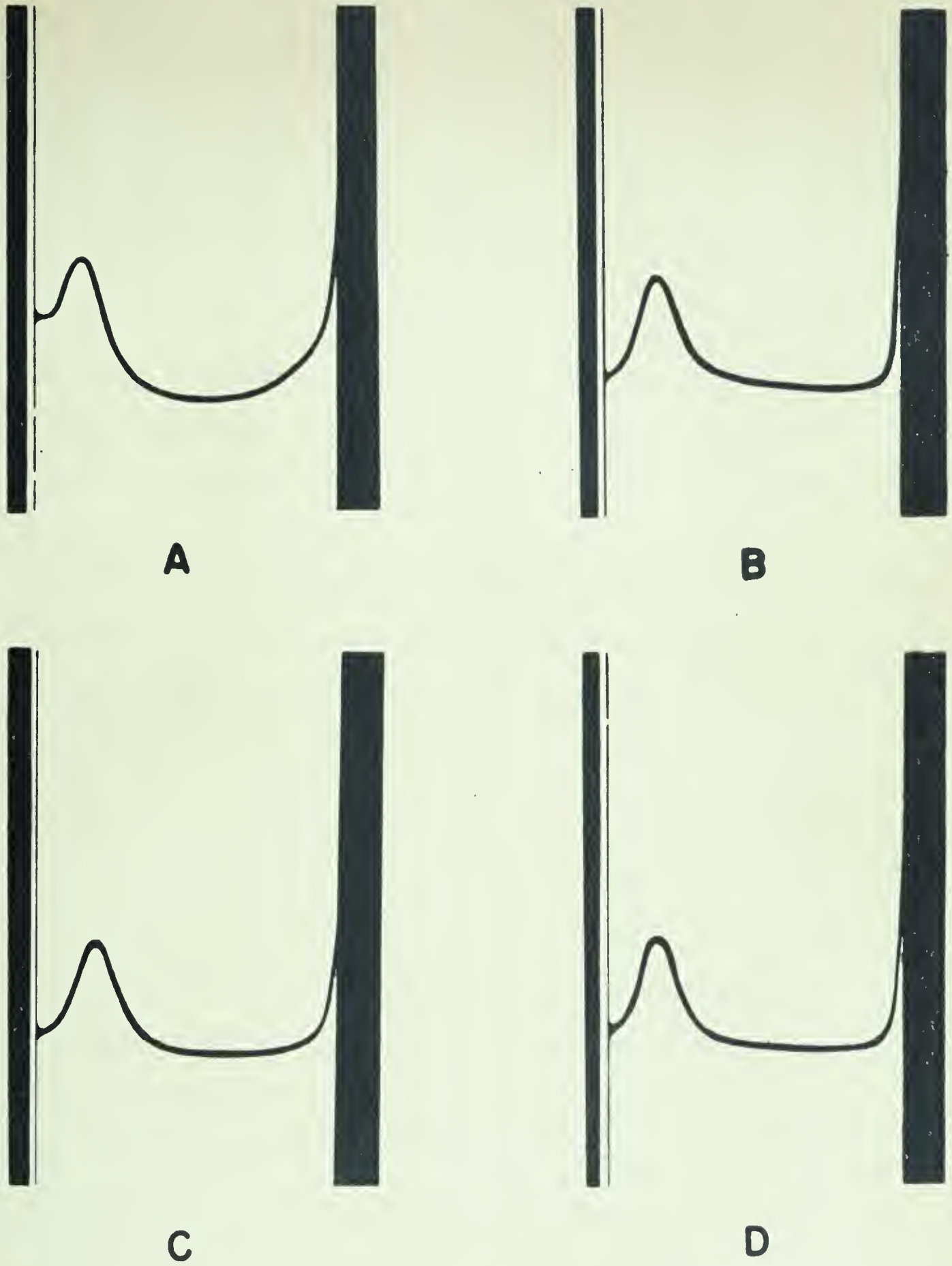


Figure 4.

Sedimentation velocity diagrams of gluten dispersed in A, sodium salicylate; B, 0.1 M lactic acid + 0.01 sodium chloride; C, 0.02 M aluminum lactate; D, 0.04 M aluminum glycolate.

concentration using Figure 3, assuming that the concentration dependence is identical in each case.

TABLE V

Intrinsic Sedimentation Coefficient of Crude Gluten

	Air	Nitrogen
Red Bobs	2.19×10^{-13}	2.14×10^{-13}
Lemhi	2.10	2.19
Mindum	2.14	2.30

Differences among the sedimentation coefficients of the glutens from the three wheats are very small as are the differences between the samples prepared under nitrogen and in air. The slight increase in the sedimentation coefficient for those dispersions prepared under nitrogen over those prepared in air may be considered insignificant, but this indication of a slight change in the particle size is consistent with viscosity studies in which intrinsic viscosities were found to be greater for dispersions prepared under nitrogen.

Figures 5 D, E and F show the sedimentation diagrams of the gluten fractions soluble in 4% salicylate, dispersed in 8% salicylate. Again, there are no differences in the diagrams among the three fractions. Sedimentation coefficients were calculated to be:

Red Bobs	2.06×10^{-13}
Lemhi	2.09
Mindum	2.13

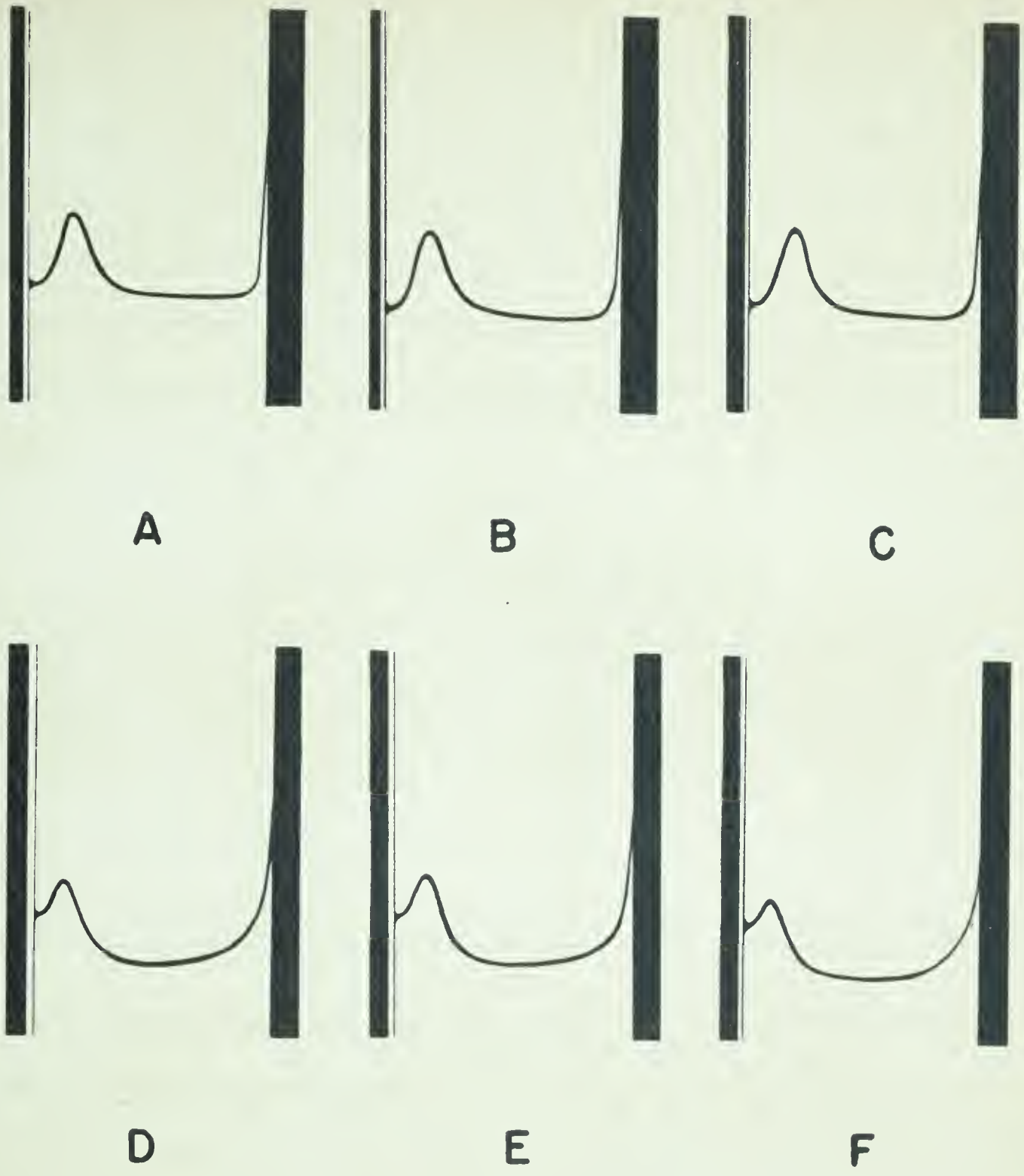


Figure 5.

Sedimentation velocity diagrams: gluten from A, Red Bobs; B, Lemhi; C, Mindum in 0.1 M lactic acid + 0.01 M sodium chloride, and gluten fraction from D, Red Bobs; E, Lemhi; F, Mindum in 8% sodium salicylate.

The effect of various treatments of gluten was also studied by sedimentation velocity. Diagrams for gluten samples oxidized with performic acid and potassium bromate, and reduced with sodium sulfite and thioglycolic acid are presented in Figure 6. Oxidation with performic acid affects the gluten from the three wheats as reflected in the sedimentation coefficients. The other treatments appeared to have no effect on the sedimentation coefficient on gluten from Red Bobs flour so that runs with the other two glutens were not made.

TABLE VI

Corrected Sedimentation Constants

Solvent	8% Na Sal	0.1 lactic acid + 0.01 NaCl		
Treatment	performic acid	$SO_3^=$	BrO_3^-	thioglycolic acid
Red Bobs	1.80	2.14	2.15	2.14
Lemhi	1.65	-	-	-
Mindum	1.33	-	-	-

The peak in the sedimentation diagram for gluten from Mindum wheat, oxidized with performic acid, Figure 6 C, is higher due to higher concentration. This higher concentration is not sufficient to account for the lower $s_{20,w}$ value, however.

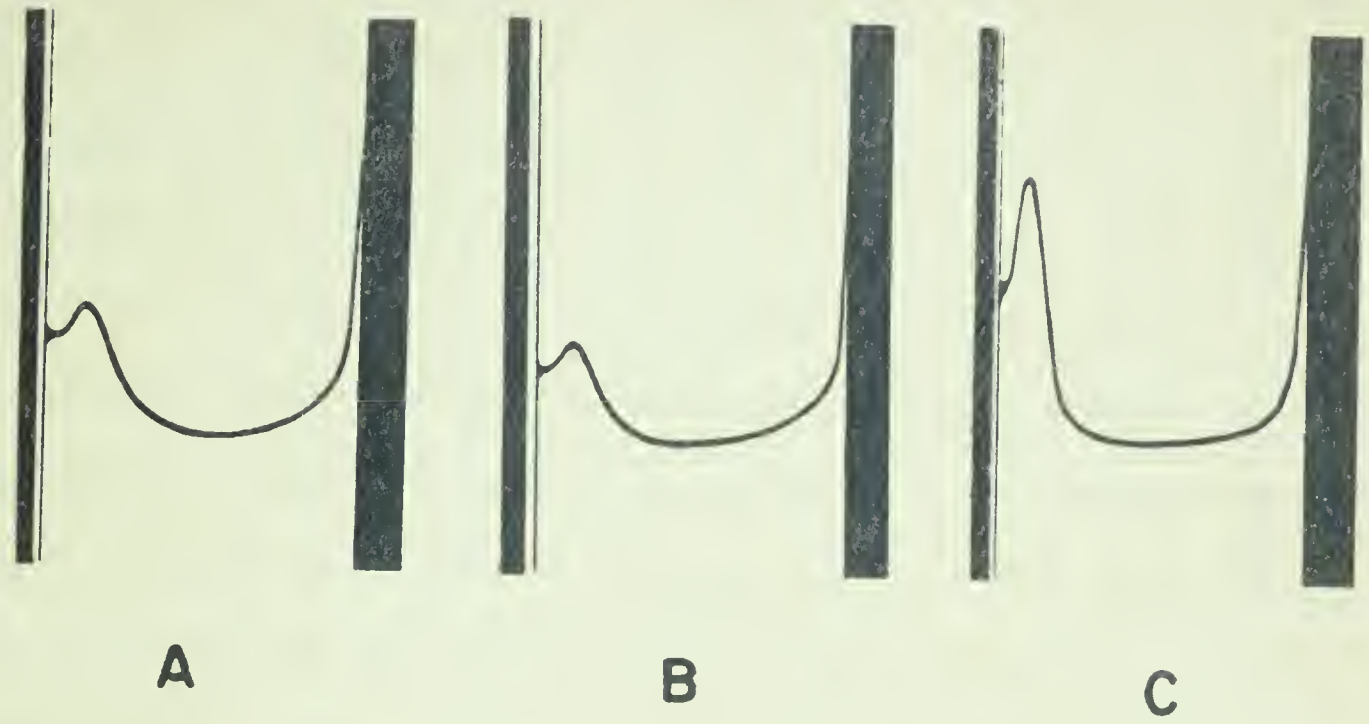


Figure 6

Sedimentation velocity diagrams. Gluten from A - Red Bobs; B - Lemhi; C - Mindum treated with performic acid, dispersed in 8% sodium salicylate; gluten from Red Bobs treated with D - bromate; E - sulfite; F - thioglycolic acid.

Molecular weight determination

The approach to sedimentation equilibrium method was used for molecular weight calculations of the fractions from the three glutens. At least six runs were made with each fraction. Calculations were made at the meniscus and at the bottom and the average values are reported. The solvent used in all cases was 8% sodium salicylate. High schlieren angles of 82.5° to 87.5° were used as lower angles gave diagrams in which the curve at the cell bottom was almost tangential so that the height of the curve was almost impossible to measure. A second run of the same solution was made in a synthetic boundary cell to enable calculation of c_o. The diagrams were magnified with a slide projector, a magnification factor of about 45 being used. This factor varied from diagram to diagram and was calculated by measuring the distance between the reference holes on the magnified diagram, and dividing by 1.6, the actual distance between the reference holes in the counterweight. A typical diagram obtained in the approach to sedimentation equilibrium is shown in Figure 7.

Molecular weights for the three fractions were calculated to be 54,200 for Red Bobs, 57,100 for Lemhi and 49,100 for Mindum.

A sample calculation of molecular weight is shown in Table VII. n is the number of intervals, starting from the meniscus where n = 0, to the point where the curve is tangential to the horizontal base line. R_n is the distance in cm. from the reference mark to the interval in question. F is the magnification factor. Z_n is the vertical distance between the curve and the horizontal base line. x_n is the distance in cm. from the axis of rotation to the interval in question. A value of 0.700 was taken for the partial specific volume (63).

TABLE VII

Molecular Weight Calculation

Sample: Mindum Fraction
Magnification factor: 41.875
Schlieren angle: 87.5°
Rotor speed: 15,220 r.p.m.

Temperature: 293.2 °K
$\bar{v}$: 0.700

n	R_n	R_n/F	x_n $(5.72^{R_n/F})$	x_n^2	Z_n	$x_n^2 Z_n$
0	24.70	0.59	6.31	39.816	3.10	123.430
1	25.12	0.60	6.32	39.942	2.60	103.849
2	25.54	0.61	6.33	40.069	2.15	86.148
3	25.96	0.62	6.34	40.196	1.65	66.323
4	26.38	0.63	6.35	40.323	1.30	52.420
5	26.80	0.64	6.36	40.450	0.95	38.428
6	27.22	0.65	6.37	40.577	0.70	28.404
7	27.64	0.66	6.38	40.704	0.45	18.317
8	28.06	0.67	6.39	40.832	0.28	11.433
9	28.48	0.68	6.40	40.960	0.20	8.192
10	28.90	0.69	6.41	41.088	0.16	6.574
11	29.32	0.70	6.42	41.216	0.12	4.946
12	29.74	0.71	6.43	41.345	0.08	3.308
13	30.16	0.72	6.44	41.474	0.06	2.488
14	30.58	0.73	6.45	41.603	0.04	1.664
						555.924

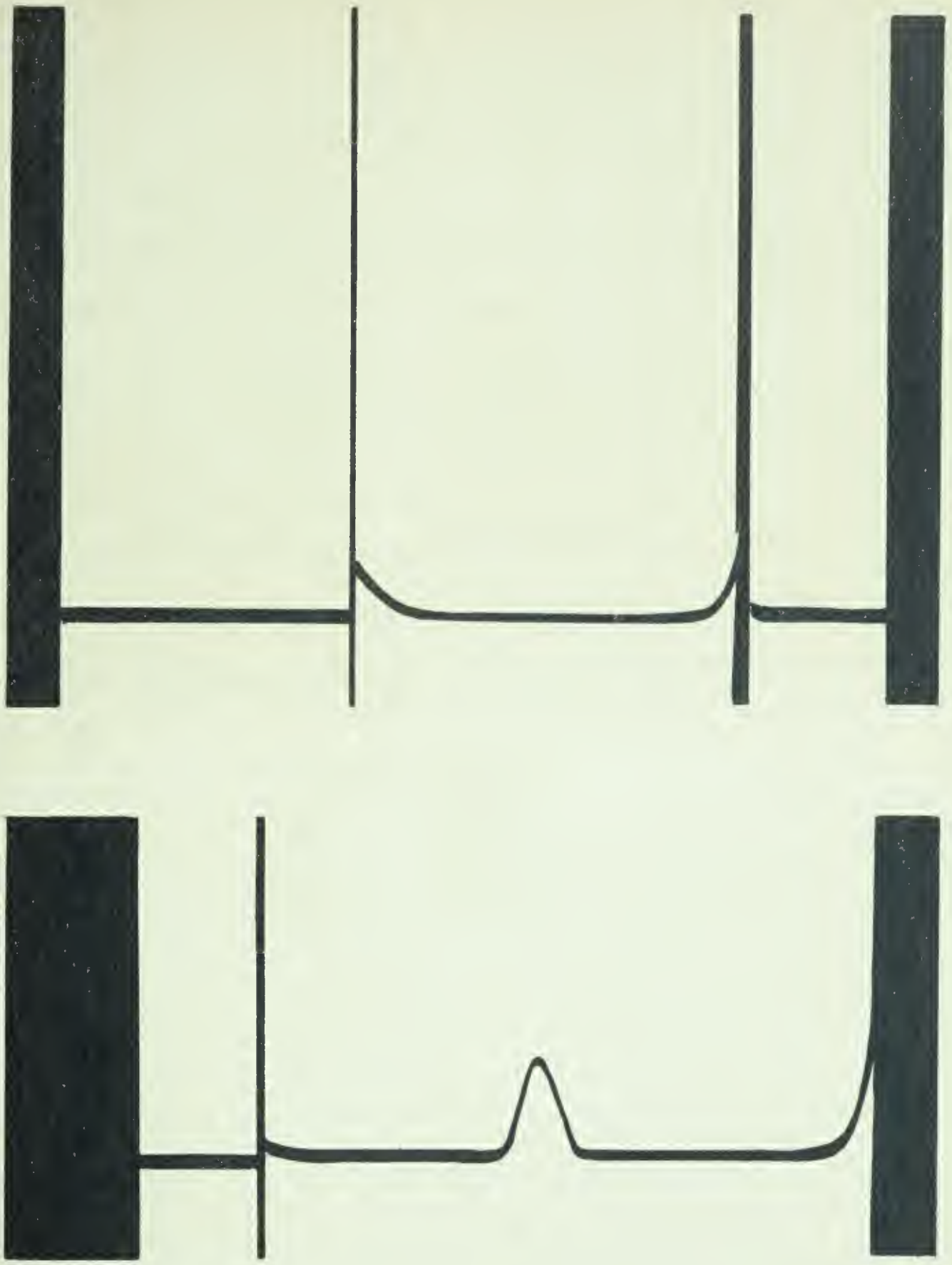
$$c_m = c_o - \frac{1}{x_m^2}\frac{dx}{F}\sum_{n=0}^{n=14} x_n^2 Z_n \qquad \left(\frac{dc}{dx}\right)_m = 3.10$$

$$c_m = 0.4838 - \frac{1}{39.816}\ \frac{0.42}{41.875}\ (555.924) = 0.3438$$

$$M = \frac{RT}{(1-\bar{v}\rho)\omega^2}\frac{(dc/dx)_m}{x_m c_m} = 3.46 \times 10^4\ \frac{3.10}{(6.31)\ (.3438)} = 49{,}400$$

Figure 7

Approach to sedimentation equilibrium diagrams for sample of TABLE VII.



Diffusion Studies

Diffusion coefficients, calculated by the method of maximum ordinate, were found to agree fairly well with those of McCalla and Gralen (63). Their values ranged from 2.14×10^{-7} $cm^2/sec.$ for the aggregated high molecular weight fraction to 4.83×10^{-7} for the most soluble fraction of molecular weight 44,000. The diffusion coefficients in the present study were calculated to be 3.76×10^{-7} $cm^2/sec.$ for the Red Bobs fraction, 3.58×10^{-7} for Lemhi and 4.08×10^{-7} for Mindum. Molecular weights, calculated from sedimentation velocity and diffusion, were found to be slightly lower than those calculated by the approach to sedimentation equilibrium method. A value of 0.700 was used for the partial specific volume (63). Molecular weights were calculated to be:

Red Bobs	48,100
Lemhi	51,300
Mindum	45,800

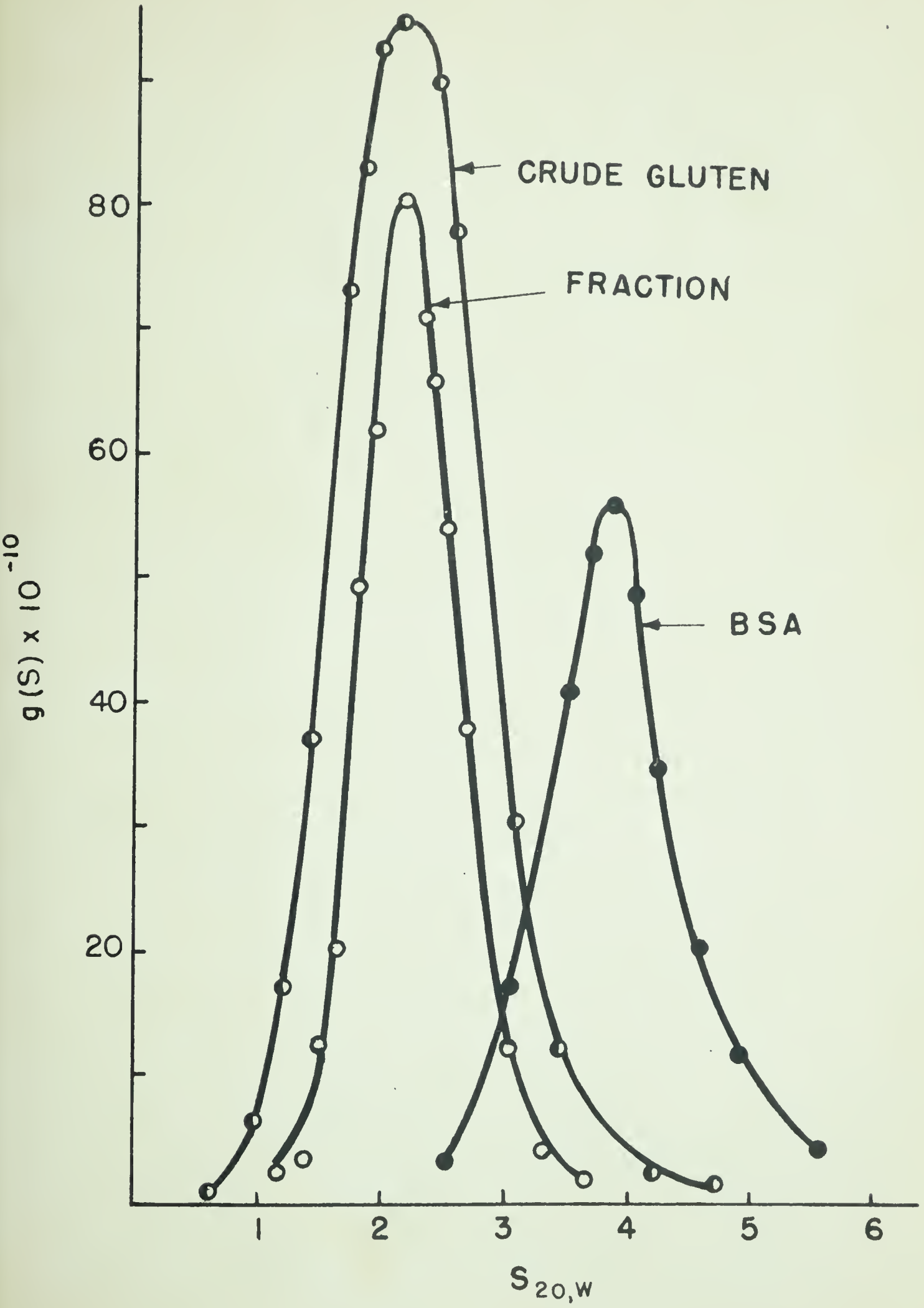
Distribution Studies

Sedimentation velocity diagrams, converted to distribution of sedimentation coefficients and corrected for diffusion are presented in Figure 8. The curve for bovine serum albumin is included to show the type of curve obtained with a fairly pure protein. The general shape of the curves is very similar to those reported for polystyrene by McCormick (66). The difference in the height of the curve is due to the concentration, since the distribution function, g(s), is proportional to the total concentration. The fraction was found to have a narrower distribution than had the crude gluten.

Only the distribution curves for gluten from Red Bobs are presented as the curves for the other two glutens were almost identical.

Figure 8

Distribution curves for gluten and the fraction from Red Bobs, and for BSA.


CRUDE GLUTEN
FRACTION
BSA
80
60
40
20
g(S) x 10^-10
1
2
3
4
5
6
S20,w

Amino Acid Analysis

The most soluble fraction of gluten, not precipitated by half saturated magnesium sulfate, comprising approximately 10 to 15% of the total gluten is quite different from the most soluble gliadin fraction in amide nitrogen and in arginine content (62). In preparing the fraction, this soluble fraction was not removed by initially precipitating the crude gluten dispersion with half-saturated magnesium sulfate. Amino acid composition of crude gluten and the fraction were determined on samples prepared with and without the removal of this soluble fraction. Results are presented in Tables VIII, IX, and X.

Initial precipitation with magnesium sulfate to remove the most soluble fraction did not affect the amino acid composition to any extent in either the fraction or crude gluten. A slight decrease was found in the arginine content of the fractions from Red Bobs and Lemhi, while ammonia increased in all three. In crude gluten, removal of the soluble fraction resulted in a decrease of aspartic acid, lysine and arginine in all three.

Acid hydrolysis of protein results in an appreciable loss of cystine. Therefore cystine for the crude gluten was determined as cysteic acid. Freshly washed out gluten was oxidized with performic acid to convert disulfide and sulfhydryl groups to sulfonic acid groups. This oxidized gluten was hydrolysed with constant-boiling hydrochloric acid in the usual manner. Cysteic acid is stable to acid hydrolysis. Determined as cysteic acid, the cystine of the three glutens was found to be almost twice that determined ordin-

TABLE VIII

Amino Acid Composition of Gluten from Red Bobs

	I	II	III	IV
Lysine	1.27 ± .11 *	0.85	0.82	0.84
Histidine	1.74 ± .06	1.81	1.85	2.04
Ammonia	4.16 ± .19	4.24	4.29	4.84
Arginine	2.70 ± .14	2.60	2.86	2.62
Aspartic acid	3.24 ± .21	2.34	2.66	2.66
Threonine	2.39 ± .09	2.09	2.14	2.17
Serine	4.75 ± .13	4.32	4.57	4.41
Glutamic acid	38.43 ±1.17	38.89	42.72	43.23
Proline	12.29 ± .35	12.15	14.22	14.79
Glycine	3.29 ± .11	3.33	2.37	2.18
Alanine	2.62 ± .15	2.19	2.22	2.12
Half cystine	1.43 ± .19	1.42	1.71	1.72
Valine	3.19 ± .23	3.27	3.67	3.37
Methionine	1.50 ± .10	1.53	1.60	1.55
Isoleucine	3.00 ± .31	3.62	3.74	3.62
Leucine	6.47 ± .15	6.41	6.98	7.15
Tyrosine	3.32 ± .15	3.40	3.20	3.32
Phenylalanine	4.97 ± .16	5.19	5.83	5.68
Tryptophane	1.42		1.04	
Cystine as cysteic acid	2.40 ± .06			
Amide nitrogen	4.83		5.12	

I Crude gluten, mean of 3 determinations
II Crude gluten, most soluble fraction removed
III Gluten fraction, mean of 3 determinations
IV Gluten fraction, most soluble fraction removed

* Standard deviation calculated on 18 determinations applicable to all the determination in Tables 8,9 and 10.

Values reported as grams amino acid/ 17.5 grams nitrogen.

TABLE IX

Amino Acid Composition of Gluten from Lemhi

	I**	II	III	IV
Lysine	1.31 *	0.79	0.76	1.10
Histidine	1.85	1.90	1.89	1.84
Ammonia	4.09	4.39	4.26	4.38
Arginine	2.98	2.54	2.52	2.30
Aspartic acid	3.06	2.30	2.67	2.49
Threonine	2.38	2.34	2.08	1.94
Serine	4.56	4.44	4.53	4.69
Glutamic acid	37.31	39.78	42.00	40.00
Proline	11.48	11.74	13.81	13.96
Glycine	2.91	2.72	2.05	2.09
Alanine	2.47	2.29	2.23	2.35
Half cystine	1.53	1.53	1.80	1.93
Valine	3.86	3.55	3.67	3.48
Methionine	1.50	1.43	1.58	1.54
Isoleucine	3.68	3.80	3.88	3.82
Leucine	6.82	6.69	7.00	6.60
Tyrosine	3.27	3.19	3.06	3.04
Phenylalanine	4.97	5.18	5.54	5.64
Tryptophane	1.24		1.08	
Cystine as cysteic acid	2.38			
Amide nitrogen	4.82		5.22	

Values reported as grams amino acid/ 17.5 grams nitrogen.
* Standard deviation given in Table VIII.
** Designation of I, II, III and IV same as in Table VIII.

TABLE X

Amino Acid Composition of Gluten from Mindum

	I**	II	III	IV
Lysine	1.46 *	0.88	0.72	0.72
Histidine	1.94	2.13	1.93	2.16
Ammonia	4.38	4.96	4.25	4.50
Arginine	3.01	2.49	2.52	2.56
Aspartic acid	3.52	2.71	2.89	3.00
Threonine	2.32	2.24	2.03	2.23
Serine	4.42	4.90	4.36	4.72
Glutamic acid	39.10	40.70	43.55	43.20
Proline	12.00	11.75	13.97	14.03
Glycine	2.87	2.61	1.94	1.95
Alanine	2.52	2.18	1.97	2.18
Half cystine	1.44	1.48	1.72	1.87
Valine	3.70	3.62	3.39	3.31
Methionine	1.63	1.50	1.55	1.59
Isoleucine	3.92	4.31	3.81	4.20
Leucine	6.80	7.03	6.77	6.64
Tyrosine	3.38	3.67	3.07	3.35
Phenylalanine	5.47	5.58	6.19	6.22
Tryptophane	0.99		0.81	
Cystine as cysteic acid	2.30			
Amide nitrogen	4.87		5.45	

Values reported as grams amino acid/ 17.5 grams nitrogen.
* Standard deviation given in Table VIII.
** Designation of I,II, III, and IV same as in Table VIII.

arily, and approximately the same for all three glutens. An average of six determinations gave values of 2.40% in Red Bobs, 2.30% in Mindum and 2.38% in Lemhi.

When gluten was treated with a sulfhydryl blocking reagent, and then oxidized with performic acid, there was a slight decrease in the cystine content of Red Bobs, but none in Mindum or Lemhi. Reagents used were iodoacetamide, methyl mercuric nitrate and N-ethylmaleimide. For Red Bobs gluten, treatment with the above reagents resulted in a decrease of 0.16% of cystine. Since this value for the free sulfhydryl groups was obtained by difference, it is not as accurate as values obtained by direct titration.

To test this further, qualitative tests were carried out using p-chloromercuribenzoate. This sulfhydryl reagent was incorporated into the dough under an atmosphere of nitrogen. The gluten was washed out and dispersed in dilute acetic acid. The dispersion was dialysed extensively to remove any unreacted reagent. The dispersion was then oxidized with a 1:1 mixture of concentrated sulfuric and nitric acids and potassium permanganate, and decolorized with hydroxylamine. Mercury was determined with dithizone (88). The characteristic orange color of the mercury-dithizone complex was found with the Red Bobs sample while with the other two, the color could not be seen. Since traces of mercury are present in air and possibly in the flour samples, it may be argued that the color may not be due to mercury from p-chloromercuribenzoate. However, since the three samples were prepared and treated under identical conditions, and since a blank determina-

tion was made, the results support those obtained with cysteic acid determinations.

Free ammonia in protein hydrolysates arises mainly from the hydrolysis of glutamine and asparagine, and small amounts from the destruction of certain amino acids. In wheat gluten hydrolysates, the presence of ammonia is attributed primarily to the hydrolysis of glutamine. Determination of free ammonia, therefore, is considered to be a measure of the amount of glutamine and the ammonia is referred to as amide nitrogen.

It was found that the number of moles of ammonia determined by the amino acid analyser was consistently lower than the number of moles of glutamic acid. Furthermore, a hydrolysed sample of glutamine, found to be 99%+ pure by Kjeldahl analysis, yielded only about 80% of the theoretical amount of ammonia. Thus, it is apparent that the ammonia determination by the amino acid analyser is low.

Amide nitrogen was, therefore, determined on each hydrolysate independently by the Kjeldahl method and presented as amide nitrogen in Tables VIII, IX and X. A very mild alkali, magnesium oxide, was used by McCalla and coworkers (62, 97) for amide nitrogen determinations. The use of a strong base, like sodium hydroxide-sodium thiosulfate mixture, used ordinarily for total nitrogen determinations, was questionable since there is a possibility of destruction of amino acids, resulting in higher values of free ammonia. Three bases, sodium hydroxide-sodium thiosulfate mixture, barium hydroxide and 15% sodium carbonate were tried. For gluten hydrolysates, the amount of ammonia titrated was highest with sodium hydroxide and

lowest with sodium carbonate. However, the lowest value, i.e., with carbonate, was still higher than that from the analyser. Results are presented in Table XI.

However, for hydrolysed samples of individual amino acids, e.g., glutamine, asparagine, glutamic acid, and a mixture of glutamine, asparagine and arginine, the amount of ammonia determined by distilling the hydrolysates with carbonate and sodium hydroxide was the same, and essentially equal to that calculated theoretically. Thus, it can be concluded that all of the amide nitrogen and none of the α-amino group nor the guanido group are liberated by hydrolysis and titrated when distilled with a fairly weak base. Results are shown in Table XII. For all the amide nitrogen determinations, 15% sodium carbonate was used.

In all the analyses for ammonia by the amino acid analyser, the amount of ammonia on a molar basis was approximately 93% of the glutamic acid content. This is appreciably higher than the 80% determined for glutamine alone, but may be attributed to destruction of certain amino acids, since it is fairly well established that cystine, methionine, serine and threonine are partially destroyed during acid hydrolysis (9).

TABLE XI

Determination of Amide Nitrogen in Gluten Hydrolysate

Hydrolysate distilled with:	g NH_3/100 g. protein *
Sodium carbonate	4.30
Barium hydroxide	4.64
Sodium hydroxide	4.93
Hydrolysate determined on Amino Acid Analyser	4.13

* Protein Nx5.7

TABLE XII

Determination of Amide Nitrogen in Hydrolysed Amino Acids

Amino Acid	Hydrolysate distilled with:		Theoretical %N of total N
	Carbonate	Sodium hydroxide	
	%N of total N	%N of total N	
Glutamine	50.1	50.5	50.0
Asparagine	50.7	51.3	50.0
Glutamine + Asparagine + Arginine *	34.1	34.7	33.3

* Molar ratio of 2:2:1

DISCUSSION

In physicochemical studies of gluten and of its components, the choice of solvent is one of the most important considerations. The effect of the solvent on the structure or the configuration of the gluten proteins has not been investigated to any extent for any specific solvent. Solvents commonly used for dispersing gluten are sodium salicylate, sodium and aluminum lactate and acetic acid. Reference has often been made to the presence of aggregates of protein molecules in certain solvents.

Aggregation is the formation of larger molecules through the operation of secondary forces between smaller molecules. Use of aluminum lactate may result in the formation of aggregates of protein-aluminum complexes. It has also been suggested that sodium salicylate causes aggregation of protein molecules (73).

Naismith (73) found considerable evidence from sedimentation velocity and viscosity studies for the occurence of aggregation of proteins from groundnut dispersed in sodium salicylate. It was suggested that the salicylate ion first penetrates the protein molecule causing a modification of the internal linkages, opening up the molecule. This would lead to exposure of many more active groups which probably then react with active groups on other molecules. The effect was most pronounced at neutral pH (6 - 7) and at high ionic strengths (ca. 10).

It is quite possible that salicylate has a similar effect on gluten proteins. Viscosity studies of gluten give an indication that such aggregations do occur both with aluminum lactate and with sodium salicylate.

Since hydrodynamic properties reflect the behavior of individual molecules only at infinite dilution, experimental data obtained at finite concentrations must be extrapolated to zero concentration. Intrinsic viscosity, which is obtained by extrapolation of relative or specific viscosity to zero concentration, is essentially a measure of the effective hydrodynamic volume of the solute. This volume may be interpreted in terms of aggregation, asymmetry, hydration or ion binding.

Considering all of the intrinsic viscosity results of gluten to date (19,74,106) and the results from the present studies, interpretation might be made in terms of aggregation. The intrinsic viscosity of gluten in 0.03 M sodium lactate (106) and in lactic acid sodium chloride solution in the present studies is appreciably lower than that in aluminum lactate (19, 74). Since the only difference in the two solvents is in the cations, the difference in the hydrodynamic volume may be attributed to aggregation due to the complexing nature of the aluminum ion.

In sodium salicylate the intrinsic viscosity of gluten was found to be comparable to that in aluminum lactate. If the interpretation of Naismith (73) is valid, then aggregation of the gluten proteins may occur. Furthermore, Colvin and McCalla (17) suggested on the basis of electrophoretic and diffusion studies of gluten in sodium salicylate, that gluten molecules are not highly solvated nor highly asymmetric. However, in both aluminum lactate and sodium salicylate, the possibility of hydration, ion binding or an increase in asymmetry due to the presence of aluminum or salicylate ions, giving rise to a greater hydrodynamic volume, cannot be entirely eliminated.

It appears that the components contributing to aggregation occur in the more insoluble gluten fraction. If salicylate causes the gluten protein, to unfold and thus to expose active groups, as suggested by Noismith (73), then the degree of unfolding is minimal with the more soluble fraction, or there are fewer active groups.

In the absence of atmospheric oxygen, the hydrodynamic volume was found to be greater in all cases. It is possible that oxygen may react with the active group and prevents reaction with active groups on other molecules. The active group may be the sulfhydryl group which might be oxidized to any of the possible oxidation states by oxygen. This would prevent the reaction of sulfhydryl groups to form inter- or intrachain resulfide bonds.

With the fractions, the slight differences in the intrinsic viscosities between dispersions prepared under nitrogen and in air, might be interpreted as indicating fewer active groups or unfolding of the molecules to a lesser extent than in the case of crude gluten.

The almost constant value of intrinsic viscosities for Mindum gluten and its fraction in sodium salicylate and in lactic acid-sodium chloride indicate that the hydrodynamic volume undergoes very little change. This might also be explained on the basis of active groups. If the active groups are the sulfhydryl groups, the results indicate that there are very few, if any, free sulfhydryl groups.

The effect of sodium sulfite on the intrinsic viscosity was found to be greatest for gluten from Mindum wheat. Assuming, as is generally done, that sulfite reduces the disulfide bonds to

S-sulfonate and to sulfhydryl groups, the greater decrease in intrinsic viscosity for Mindum gluten might be attributed to a larger number of intermolecular disulfide bonds being reduced. When all the experimental results of the present investigation are considered, this conclusion appears reasonable.

Intrinsic viscosities and molecular weights of other well-characterized proteins as given by Tanford (105), are presented in Table XIII for comparative purpose and to show the asymmetry of gluten protein molecules.

The intrinsic viscosities of the first four proteins approach the theoretical minimal value of 0.025. This value was derived from theoretical considerations by Einstein for particles which were compact spheres (reviewed by Tanford, (105)). Thus, these four proteins remain in solution as compact spheres. The other four, on the other hand, deviate considerably from a spherical shape as indicated by large intrinsic viscosities. Deviation from the theoretical value of 0.025 is explained by asymmetry and/or solvation. The gluten fraction used in this study and gliadin, with an intrinsic viscosity of about 0.16 dl/g and a molecular weight of approximately 45,000 must be asymmetric and/or solvated.

Sedimentation velocity studies of gluten have consistently shown only one peak regardless of the dispersing media (63, 65). This was again shown to be the case in the present study. Even with the addition of sulfite, thioglycolic acid and bromate, there was no change in the sedimentation pattern or in the sedimentation coefficient.

TABLE XIII

Intrinsic Viscosities and Molecular Weights
of Well Characterized Proteins

	dl/g	Molecular Weight
Ribonuclease	0.033	13,683
β-lactoglobulin	0.034	35,000
Serum albumin	0.037	65,000
Catalase	0.039	250,000
Tropomyosin	0.52	93,000
Fibrinogen	0.27	330,000
Collagen	11.50	345,000
Myosin	2.17	493,000

For gluten proteins, it was found that changes in the hydrodynamic properties were more readily detected with viscometry than with sedimentation velocity. Treatment of gluten with sulfite, for example, showed no change in the sedimentation coefficient, while an appreciable decrease was detected in intrinsic viscosity. McCalla and Verma (65) also reported that no change in the sedimentation coefficient was observed when ~~when~~ gluten was treated with bisulfite.

The almost constant $s_{20,w}$ values obtained for gluten and the fraction may be explained on the basis of aggregation. It is possible that there is a varying degree of aggregation of the gluten molecules. The extent of aggregation may be minimal in the more soluble fraction of gluten and maximal in the more insoluble glutenin fraction. When subjected to sedimentation velocity, the heavily aggregated portion in crude gluten dispersions is sedimented out, leaving in dispersion, molecules or molecular aggregates predominant in the fraction. This is visible on the ultracentrifuge screen before full speed is reached. A small peak is usually formed before the appearance of the major peak for crude gluten dispersions. The minor peak, however, soon disappears. Sedimentation coefficients for the fraction and crude gluten, therefore, would be almost identical.

A possible explanation for the effect of sulfite detectable in viscometry, but not with sedimentation velocity, may be that the 'heavily aggregated' portion is primarily affected by the reducing agent. Crude gluten dispersions studied viscometrically contained these aggregates, and changes in the aggregates due to the action of the reducing agent was reflected in the intrinsic viscosity.

Results of Nielsen et al. (74) may be used to substantiate this suggestion. They reported that cleavage of all the disulfide bonds by reduction with sulfite or by oxidation with performic acid resulted in a decrease in intrinsic viscosity from 0.68dl/g to 0.22 dl/g, as well as a decrease in molecular weight from several million to 20,000. The intrinsic viscosity of gliadin, on the other hand, was not affected when subjected to performic acid oxidation. It appears, therefore that the components which are affected by sulfite and by performic acid are present only in the more insoluble glutenin fraction and not in gliadin or the fraction which was used in the present studies.

The effect of sulfite on gluten might perhaps be detected by sedimentation velocity at a lower speed, in which case the heavily aggregated portion will not immediately sediment out.

There is still a great deal of uncertainty regarding the molecular characteristics of gluten proteins. Nielsen et al. (74) suggested that glutenin has a basic peptide unit of molecular weight 20,000. The molecular weight of gliadin has been reported in the range from 25,000 to 50,000.

Jones et al. (51) reported a weight average molecular weight of 2 - 3 million for glutenin but reported that their preparations also contained small molecules of molecular weight approximately 50,000. The molecular weight of impure gliadin was reported to be about 200,000and that of a purified preparation between 40,000 - 50,000. As suggested by McCalla and Verma (65), if gluten is made up of species of molecular weights 2,000,000, 200,000 and 50,000, sedimentation velocity analysis should certainly separate such widely differing

components, unless the degree of hydration and asymmetry are extremely large. Frictional ratios for gluten reported by Colvin and McCalla (17) and McCalla and Gralen (63) indicate that gluten molecules are not very highly hydrated nor very highly asymmetric. Jones (51) suggested that the observed molecular weights were real and resulted from chemical bonds rather than physical aggregation. This is questionable and more work is required to resolve this problem.

The recent paper of Taylor and Cluskey (106) deserves some consideration. Viscosity, diffusion and sedimentation studies were reported and all three require evaluation. For intrinsic viscosity determinations, two concentrations of sodium lactate were used. In the lower ionic strength solvent, 0.003, the intrinsic viscosity of gluten and glutenin were much higher. The higher values were attributed to the glutenin component, since gliadin was not affected greatly by a change in ionic strength. According to Tanford (105), the electrostatic effect of protein is not as great as believed formerly, so that a high concentration of electrolyte is not required to douse the effect. At ionic strength of 0.01 the charge effect was reported to be small in most cases. However, it is pointed out that only at reasonably high ionic strengths can the charge effect be neglected in viscosity, diffusion and sedimentation studies.

It appears, therefore, that the higher values obtained with 0.003 ionic strength solution may be due to charge effects and not due to any component of gluten as suggested by Taylor and Cluskey (106). The intrinsic viscosity of gliadin, however, did not

change appreciably upon changing the solvent from 0.03 ionic strength to 0.003. This might be explained on the basis of ionizable groups on the molecule. The number of such groups may be very much less on gliadin proteins than on the more insoluble fractions so that the charge effect is less pronounced. Interpreting the data obtained at low ionic strength in terms of basic structure of gluten seems questionable.

Diffusion coefficients determined by the same workers (106) in sodium lactate are lower than those reported previously (58, 63). Values are reported at 1°C and the data to correct to the standard value $D_{20,w}$ are not given. Using a rough estimate of 1.8 for the correction factor (the principal factor being the correction for density of water at 1°C to that at 20°C) diffusion coefficients would be 1.74×10^{-7} for gluten, 3.78×10^{-7} for gliadin and 0.95×10^{-7} for glutenin. These values are low when compared with the results of McCalla and Gralen (63) who reported values of 2.14×10^{-7} for the high molecular weight function (corresponding roughly to the glutenin) and 4.83×10^{-7} for the most soluble fraction of molecular weight 44,000 (corresponding to gliadin).

Molecular weights calculated from "the peak of the sedimentation curve and the diffusion coefficient" were reported to be 1,000,000 for glutenin, 100,000 for gluten and 60,000 for gliadin. Presumably the standard equation

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

was used for these calculations. Sedimentation coefficients are not presented but calculation from the above equation using the value given for D, yields a sedimentation coefficient of 1.15 x

10^{-13} for gliadin. Such a low value has not been reported to date. Yet, from the distribution curve presented, the predominating species in gliadin is shown to have a sedimentation coefficient of about 3. The molecular weight for gliadin is higher than any reported to date, probably due to the low value of the diffusion constant.

The significance of the analysis of the sedimentation diagram for distribution of sedimentation coefficients by Taylor and Cluskey (105) is questionable. In order to calculate the true distribution curve, corrections for the spreading of the sedimentation diagram with time due to diffusion and the narrowing due to the concentration dependence of the sedimentation coefficient, must be made. Neither was taken into account here. The distribution curves when corrected for the two effects should be narrower. An excellent example of the effect of diffusion is presented by McCormick (66) in Fig. I for polystyrene. Since it was found in the present study that the sedimentation coefficient was concentration dependent in lactic acid-sodium chloride solution, correction for this effect is also necessary.

It must be concluded that the results in this paper (106) are of questionable value since many factors of significance have been neglected.

Molecular weights and diffusion coefficients determined in this study are quite similar to those of McCalla and Gralen (63). The method of the approach to sedimentation equilibrium is not as accurate as the sedimentation equilibrium method for gluten proteins dispersed in salicylate. Even when high schlieren diaphram angles are used it is difficult to measure the height of the curve at the meniscus and at the cell bottom precisely.

Results indicate that there are very small differences in the molecular weights of the gluten fractions from the three wheats in 8% sodium salicylate. The slight differences both in diffusion coefficients and in molecular weights cannot be definitely considered significant.

The relationship between the sedimentation coefficient and the molecular weight has been empirically derived for most polymers with which distribution of sedimentation coefficient studies have been made. Thus, it is possible to obtain the distribution of molecular weight of the sample. However, for gluten the molecular weight cannot be related to the sedimentation coefficient due to the uncertainty in the molecular weight. Distribution studies are useful, though, in giving an indication of the range of sedimentation coefficients for the various components.

The narrow distribution curves obtained for both gluten and the fraction indicate that there is a narrow range of sedimentation coefficients with the principal component in both having a sedimentation coefficient of 2.1×10^{-13}.

It was suggested earlier in the discussion of sedimentation velocity experiments that the components of the insoluble glutenin fraction have a greater tendency to form aggregates and that these aggregates are sedimented out before full speed on the ultracentrifuge is attained. Sedimentation coefficients of both gluten and the fraction were, therefore, the same. Most of the aggregated components of high sedimentation coefficients are absent from the solution so that the resultant distribution curve is very similar to

that of the fraction. However, the presence of some components with higher sedimentation coefficients is indicated by the distribution curve which is slightly broader than that of the fraction.

Fractionation by dilution removes the insoluble glutenin components so that the distribution curve is narrow. That the breadth of the curve is comparable to that of bovine serum albumin cannot be interpreted as conclusive in showing the homogeneity of the gluten fraction. There is an indication that, in sodium salicylate, the components in the gluten fraction are fairly homogeneous in size.

For crude gluten dispersions, however, it appears that a better indication of the distribution of sedimentation coefficients might be obtained if sedimentation velocity runs were made at lower speeds to prevent the heavier particles from sedimenting out. On the basis of the results it can be said only that, under the conditions of the experiment, the components remaining in solution in crude gluten dispersions appear to be fairly homogeneous.

Distribution studies in other solvents, e.g. sodium lactate, with proper corrections may yield further information on the dispersed gluten.

The total recovery of amino acids, calculated on the basis of a theoretical nitrogen content of 17.5%, in all cases amounted to more than 100%. It was found that the determination of nitrogen by the Kjeldahl method was critical in calculating percentages of amino acids. Although triplicate and sometimes quadruplicate nitrogen determinations were made on each hydrolysate, the error in this de-

termination is a contributing factor in percentages of amino acids greater than 100%.

The factor of 5.7 was used for converting the amount of nitrogen to protein since it was found by Osborne (77) and many later workers that the nitrogen content of flour protein and gluten was 17.5%. All data on amino acid composition by other workers have been converted to this basis when values were reported as grams of amino acid/16 grams nitrogen.

Amino acid analysis of the three wheat glutens and the fractions show that there are very little, if any, differences among the glutens as well as among the fractions. There are differences between the fraction and crude gluten in all three classes. Lysine content is lower, while glutamic acid, proline and cystine are higher in the fraction. These differences have been known since amino acid analysis was begun.

Standard deviations for each amino acid were calculated by a commonly used statistical method. The large deviations found for valine and isoleucine may be attributed to the time of hydrolysis. It has been established (9) that valine and isoleucine increase with increased hydrolysis time. Although the time of hydrolysis was kept fairly constant, the amount of these two amino acids hydrolysed may not be constant unless a longer hydrolysis time is used.

The most soluble fraction of gluten, which presumably corresponds to the so-called water soluble proteins of wheat flour and which is retained in the gluten complex during the gluten washing

process, has a different amino acid composition. It has been shown by Woychik et al. (115) that this water soluble fraction is lower in glutamic acid, proline and ammonia, and higher in arginine and cystine. The differences in amide nitrogen and in arginine have been shown by McCalla and coworkers (62, 97). The amino acid composition of gluten and the fraction with and without the removal of this soluble fraction differed in arginine and ammonia contents. Removal of the soluble fraction caused an increase in ammonia and a decrease in arginine in all three glutens and their fractions. Surprisingly, glutamic acid was not affected. If ammonia arises primarily from the hydrolysis of glutamine, there should be an increase in glutamic acid corresponding to the increase in ammonia. A possible explanation is that not all of the glutamic acid occurs as the amide in the water soluble fraction. Removal of this fraction would, thus, increase the ratio of amide nitrogen to total nitrogen. Therefore, total ammonia content in the hydrolysate would increase.

Determinations of amide nitrogen by the Kjeldahl method as shown in Tables VIII, IX and X were higher than those determined by the amino acid analyser. Why the analyser does not detect all the free ammonia in the hydrolysate is not known. Amide nitrogen by Kjeldahl determination amounts to about 110% of the glutamic acid content on a mole for mole basis for all the samples. It must be borne in mind that serine, threonine, methionine and cystine are partially destroyed during hydrolysis so that ammonia would be one of the products of destruction. Furthermore, aspartic acid may be present as asparagine.

That cystine is destroyed during hydrolysis is clearly demonstrated by the results of cystine determined as cysteic acid. Had hydrolyses been done for different lengths of time, it would have been possible to show progressive decreases in threonine, serine and methionine. The values presented for these amino acids are uncorrected. For comparative purposes, however, these values are satisfactory.

Determination of cystine as cysteic acid showed that in all three glutens the cystine content was approximately the same. However, the ratio of sulfhydryl to disulfide groups in the gluten from the three flours appears to differ. Determination of free sulfhydryl groups by the method described, indicated that about 7% of the total sulfur (i.e. sulfur determined as cysteic acid) occurs as free sulfhydryl groups in gluten from Red Bobs flour, while very little, if any, was present in gluten from either Lemhi or Mindum flours. In flour, it has been reported by Bushuk (14) that slightly more free sulfhydryl groups are present in Mindum flour than in hard wheat flours. Results are not comparable since his were determined and flour and those reported here on gluten.

An attractive explanation can be offered for one possible factor in the differences in the basic properties, which may be related to the quality of the flours for bread making purposes. If the difference in the sulfhydryl content in the three glutens is real, then it may be suggested that the ratio of free sulfhydryl to disulfide groups is one factor which determines the rheological characteristics of the flours. With free sulfhydryl groups present,

an exchange reaction as suggested by Jensen (46) can take place. Goldstein (34) suggested that such an exchange reaction occurs in dough since the rheological properties were affected when the dough was treated with a sulfhydryl blocking reagent. If there are only few sulfhydryl groups, as indicated by the results for glutens from soft and durum glutens, very little, if any, sulfhydryl-disulfide exchange reaction is likely to occur.

Interpretation on this basis can be extended to the response of the three glutens to physical manipulations. Generally, glutens from hard wheats are elastic and can be stretched into thin sheets, while those from soft and durum wheats are very extensible and "flowy", but cannot be formed into thin sheets. From physical manipulations, one can conclude that there are basic differences in the physical constitution among the three glutens. The exact role of the sulfhydryl groups cannot be specified, but the difference between the physical characteristics of gluten from hard wheat and those of gluten from the other two wheats might be attributed partly to the sulfhydryl groups. Another factor contributing to these differences may be the location of the disulfide bonds, i.e., whether they are inter- or intramolecular.

Performic acid oxidation causes a pronounced reduction in the sedimentation coefficients of the three glutens. Gluten from Mindum is affected most, that from Lemhi less, and that from Red Bobs least. These changes may be explained on the basis of disulfide bonds. If the majority of the disulfide bonds in gluten from Mindum are intermolecular, then oxidation of these bonds with performic acid would

cause a degradation of the protein particles into smaller units. On the other hand, if the disulfide bonds are intramolecular, oxidation will cause unfolding but will not break the molecule into smaller units. The marked decrease in the sedimentation coefficient of gluten from Mindum may be interpreted as indicating the presence of intermolecular disulfide bonds. On the same line of reasoning, it may be suggested that there is a greater proportion of intramolecular disulfide bonds in gluten from Red Bobs than that in Lemhi, which in turn, might have fewer intermolecular disulfide bonds than the gluten from Mindum.

This is only one possible interpretation. Lowering of the sedimentation coefficient may also reflect the rupture of intramolecular disulfide bonds resulting in a change of shape. In this case, the sedimentation coefficient will decrease while the intrinsic viscosity will increase due to a more random structure. If, however, the results of Nielsen et al. (74) are valid, i.e., that intrinsic viscosity decreased markedly from 0.68 dl/g to 0.22 dl/g and the molecular weight decreased from 2 - 3 million to 20,000 after performic acid oxidation, then interpretation in terms of intermolecular disulfide bonds might be acceptable.

SUMMARY

Glutens from three classes of wheat, soft, hard and durum were studied by physical and chemical methods. Sedimentation velocity studies on gluten and on a gluten fraction, soluble in 4% sodium salicylate, indicate that there are no differences among the glutens as judged by the sedimentation coefficients. Diffusion experiments, molecular weight determinations by the approach to sedimentation equilibrium and by a combination of diffusion and sedimentation velocity, and distribution of sedimentation coefficient studies indicate very small differences in the mean particle size. Intrinsic viscosity determinations showed slight differences in the effective hydrodynamic volumes of the three glutens.

Amino acid analysis showed that there were essentially no differences among the three glutens. Determination of the total disulfide-sulfhydryl content as cysteic acid by performic acid oxidation showed no difference in the three glutens.

It is difficult to detect differences in the physical properties of glutens from the three classes of wheat on the basis of diffusion and sedimentation (velocity, approach to sedimentation equilibrium and distribution analysis) studies. Intrinsic viscosities, interpreted in terms of the effective volume, indicate differences between hard and durum wheat glutens, the volume of durum gluten being smaller. The effect of reducing agents is most pronounced in durum gluten indicating a difference in structure with respect to the disulfide bond.

The greatest change in the behavior of gluten was observed after the gluten from the three wheats was oxidized with performic acid. Gluten from Mindum was affected to the greatest extent as reflected in the reduction of the apparent sedimentation coefficient and gluten from Red Bobs was affected the least. It is suggested that one factor giving rise to the difference in physical properties of gluten may be the number of intermolecular disulfide bonds.

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